Evaluation of Thymoquinone-induced Apoptosis

In Non-Small Cell lung Cancer Cell line (A549): An in vitro study

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN
BIOTECHNOLOGY

Submitted By

Mr. Nibedan Ghatak
Registration ID: 230020271252
Enrollment ID: 2321210002027

Advisor
Dr. Arghya Adhikary Assistant
Professor
Department of Life Science and Biotechnology Jadavpur
University
Kolkata

School of Health Science and Translational Research Sister
Nivedita University
Kolkata

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যাদবপুর বিশ্ববিদ্যালয়
কলকাতা-৭০০০৩২, ভারত

*JADAVPUR UNIVERSITY
KOLKATA-700 032, INDIA

DEPARTMENT OF LIFE SCIENCE AND BIOTECHNOLOGY

CERTIFICATE

This is to certify that the project report entitled "**Evaluation of Thymoquinone-induced apoptosis in Non-Small Cell Lung Cancer Cell LineA549: An Invitro Study"** submitted by **Mr. Nibedan Ghatak** for the partial fulfilment of the degree of Masters of Science to the **Department of Biotechnology, School of Health Science and Translational research, Sister Nivedita University** has completed its period as a long-term trainee in the area related to **lung cancer,** at the **Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India,** from **8th Jan 2025, to 25th May 2025** under the supervision of **Dr. Arghya Adhikary.**

Nibedan is a very methodical and sincere research worker and can maintain cordial relations within the laboratory. He has learnt many sophisticated techniques required for modern research in the field of Cancer biology and Cell signalling. I wish him all success in life.

Arghya Adhikary. 25/5/25.

Dr. Arghya Adhikary
Assistant professor,
Dept of Life Science and Biotechnology
Jadavpur University,
188, Raja Subodh Chandra Mallick Rd, Jadavpur,
Kolkata, West Bengal 700032
***Email**: arghyaa.lifesc@jadavpuruniversity.in*
Ph: 9830428550

Dr. Arghya Adhikary
Assistant Professor
Dept. of Life Science and Biotechnology
Jadavpur University
Kolkata - 700 032

DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**Evaluation of Thymoquinone-induced Apoptosis in Non-Small Cell Lung Cancer Cell line(A549): An *in vitro* study**: in partial fulfilment of the requirements for the award of the degree of Master of Science in biotechnology is an authentic record of my own work carried out during the period **from 8th January 2025 to 25th May 2025** under the supervision of **Dr. Arghya Adhikary** Department of Life Science and Biotechnology, Jadavpur University, Jadavpur, Kolkata, Weste Bengal.

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Nibedan Ghatak
05/06/2025

(Signature of the students with Date)

Name of the Student- Nibedan Ghatak
Registration no.- 230020271252
Department- Department Of Biotechnology

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DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

TO WHOM IT MAY CONCERN

This is to certify that the project report titled "**Evaluation of Thymoquinone-induced Apoptosis in Non-Small Cell Lung Cancer Cell line(A549): An *in vitro* study**", submitted by Mr. **Nibedan Ghatak** (Registration No. 230020271252 Enrolment No. 2321210002027) to the Department of Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 03.06.2025 .

Place: SISTER NIVEDITA UNIVERSITY

Signature of Head of the Department

Seal

Head
Department of Biotechnology
Sister Nivedita University
Block DG-1/2, Action Area 1
New Town, Kolkata-700 156

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ABBREVIATIONS:

1.	A549	Adenocarcinomic human alveolar basal epithelial cells
2.	SCLC	Small cell lung cancer
3.	NSCLC	Non small cell lung cancer
4.	TQ	Thymoquinone
5.	Bax	Bcl-2-associated X protein
6.	Bcl	B-cell lymphoma
7.	BSA	Bovine serum albumin
8.	B(a)P	Benzo(a)pyrene
9.	NF-κB	Nuclear factor kappa B
10.	TNF-α	Tumor necrosis factor-a
11.	mRNA	Messenger Ribonucleic Acid
12.	ERK	Extracellular-signal-regulated kinase
13.	Qu	Quercetin
14.	p-AKT	Protein kinase B

15.	mTOR	Mammalian target of rapamycin
16.	M phase	Mitotic phase
17.	PBS	Phosphate buffer saline
18.	SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
19.	β-Actin	Beta -actin
20.	PI	Propidium iodide
21.	FITC	Fluorescein isothiocyanate
22.	PVDF	Polyvinyledene fluoride
23.	mM	Milli molar

ABSTRACT :

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, accounting for approximately 12.4% of all cancer diagnoses and ranking as the most commonly diagnosed cancer globally. Non-Small Cell Lung Cancer (NSCLC) is the most prevalent subtype and presents significant therapeutic challenges due to the side effects, toxicity, and resistance associated with conventional treatments like chemotherapy and radiotherapy. Hence, the search for biocompatible, cost-effective alternatives with minimal side effects is a growing priority.

Thymoquinone (TQ), a bioactive compound derived from *Nigella sativa* (black seed), has been extensively studied for its anti-inflammatory, antioxidant, and antineoplastic properties. The present in vitro study aimed to evaluate the anticancer potential of TQ on A549 human lung adenocarcinoma cells. A549 cells were treated with varying concentrations of TQ over different time durations, and their response was assessed using standard viability assays, protein expression analysis through western blotting, and flow cytometric quantification of apoptosis and necrosis via annexin V-FITC and propidium iodide staining.

The results demonstrated that TQ significantly reduced the viability of A549 cells in a dose- and time-dependent manner. Flow cytometry revealed increased apoptotic cell populations upon TQ treatment. Protein blotting data showed upregulation of p53 and Bax and downregulation of Bcl-2, indicating activation of intrinsic apoptotic pathways. Furthermore, enhanced expression of caspase-3 and caspase-9 confirmed caspase-dependent apoptosis.

These findings suggest that thymoquinone effectively promotes apoptosis in lung cancer cells and may serve as a promising therapeutic agent. However, further preclinical and clinical investigations are essential to validate its safety, pharmacokinetics, and efficacy in vivo.

Keywords

Thymoquinone (TQ); Nigella sativa; A549 lung cancer cells; Apoptosis; p53; Caspase-3; Bax/Bcl-2 ratio

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Chapter 1 :- INTRODUCTION

Lung cancer remains the leading cause of cancer-related deaths worldwide, both in terms of incidence and mortality. Despite continuous scientific advancements, the survival rate for lung cancer patients remains dismally low. In 2020 alone, approximately 1.8 million deaths were attributed to lung cancer, making up nearly 18% of all cancer deaths globally (1–4). In the United States, around 200,000 new cases of lung cancer are diagnosed every year. India accounts for 6.9% of new lung cancer cases and 5.9% of all cancers, with a significant concentration in metropolitan cities like Delhi, Chennai, and Bengaluru (5,6).

Lung cancers are broadly categorized into two major types: Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). SCLC typically originates in the bronchi, affecting the tracheal region and rapidly progressing to deeper structures, including the lymph nodes (3). SCLC comprises two subtypes: small cell carcinoma and mixed small/large cell carcinoma, with small cell carcinoma being the more prevalent subtype, accounting for around 15% of lung cancers. The predominant cause of SCLC is tobacco smoking (3,4). Due to their rapid proliferation and early metastasis, SCLCs can be targeted for quicker treatment compared to NSCLCs (7).

In contrast, NSCLCs progress more slowly and are less aggressive than SCLCs. They account for a majority of lung cancer cases and are further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (8). Adenocarcinomas originate from the outer regions of the lung and often result in mucus production. Squamous cell carcinomas typically affect the central bronchi, and large cell carcinomas can develop in any lung region, often metastasizing rapidly. Despite their slower progression compared to SCLC, NSCLCs often have poor prognosis due to their aggressive metastatic behavior and resistance to conventional therapies.To understand the basis of lung cancer cell survival and the mechanism of action of therapies, it is crucial to understand apoptosis, the natural, regulated process of programmed cell death that eliminates abnormal, damaged, or excess cells to maintain tissue homeostasis. Apoptosis occurs through two primary signaling pathways: the extrinsic and the intrinsic pathways. The extrinsic pathway is triggered by external signals, where death ligands bind to tumor necrosis factor (TNF) receptor family proteins on the cell surface, activating caspases, which are proteolytic enzymes that dismantle cellular structures (9). On the other hand, the intrinsic pathway is activated by internal stressors like DNA damage or lack of growth factors, and is tightly regulated by pro-apoptotic proteins like Bax and anti-apoptotic proteins like Bcl-2. The shift in balance towards Bax leads to mitochondrial membrane permeabilization, cytochrome c release, and ultimately caspase activation and cell death.Several proteins are integral to apoptosis, including the Bcl-2 family, p53, heat shock proteins, and amyloid-B peptide, with caspase-3 being the principal executioner caspase. It initiates the execution phase of apoptosis, leading to nuclear fragmentation, chromatin condensation, and apoptotic body formation (9).

Currently, chemotherapy remains the cornerstone of treatment for NSCLC, especially over the past three decades. The standard regimen for non-squamous NSCLC includes pemetrexed-platinum, along with drugs such as bevacizumab, paclitaxel, docetaxel, cisplatin, methotrexate, gefitinib, and porfimer (9). Combined therapies involving chemotherapy, radiotherapy, and prophylactic cranial irradiation have shown improved survival of up to 18–24 months in some patients (10). Neoadjuvant chemotherapy also shows promise in resectable NSCLC cases. However, these therapies often come with severe side effects, such as anemia, fatigue, infertility, infection, and organ toxicity—due to their non-selective targeting of both cancerous and normal cells. Similarly, while surgical techniques (like laser surgery, cryosurgery) can be effective, anesthesia complications and postoperative risks remain critical concerns (11). Despite global implementation, current treatments still leave significant gaps in efficacy and patient tolerance (12).

Thus, the search for safer, more targeted therapeutic options is ongoing. Recently, the anticancer potential of plant-derived compounds has gained considerable attention due to their low toxicity profiles and bioactive properties (13). One such promising plant is Nigella sativa, commonly known as black cumin or black caraway, a herbaceous annual plant from the Ranunculaceae family (14). It is widely cultivated across West Asia, East Africa, and the Middle East. The seeds of N. sativa have been traditionally used in medicine and are reported to possess antioxidant, antidiabetic, immunomodulatory, and anticancer effects (15). They are rich in proteins, fatty acids, carbohydrates, amino acids, alkaloids, crude fiber, along with essential minerals like sodium, calcium, potassium, iron, and water-soluble vitamins (16).The primary bioactive compound of black seed is Thymoquinone (TQ) [2-methyl-5-isopropyl-1,4-benzoquinone], first extracted by El-Dakhakhany (17). TQ has been widely studied for its anticancer, anti-inflammatory, and antioxidant properties. Techniques such as gas chromatography, high-performance liquid chromatography (HPLC), and differential pulse polarography are used for its quantification (18). Other active components of the seed include thymohydroquinone, dithymoquinone, and thymol (19). TQ’s lipophilic quinone structure enables it to penetrate cellular and subcellular membranes, targeting transcription factors and kinases involved in the cancer microenvironment. It has demonstrated effectiveness in inhibiting proliferation, migration, invasion, and angiogenesis in cancer models.TQ's anticancer potential has been tested on several human lung cancer cell lines, including A549, NCI-H146, and NCI-H460, where it was found to significantly increase levels of pro-apoptotic markers such as p53, the Bax/Bcl-2 ratio, and caspases, while inhibiting angiogenesis by downregulating Gro-alpha and ENA-78, which are key pro-angiogenic factors (20,21,22).The A549 cell line, specifically, is a widely accepted in vitro model derived from human alveolar basal epithelial cells. It serves as an excellent system for NSCLC studies due to its well-characterized phenotype, responsiveness to apoptotic stimuli, and relevance in evaluating natural compound-based therapeutics.This background thus lays the foundation for exploring the apoptosis-inducing effects of Thymoquinone in A549 cells as a potential **therapeutic approach** for non-small cell lung cancer.

Chapter 2:- Review of Literature

Thymoquinone (TQ), a naturally occurring bioactive compound, has garnered significant interest for its therapeutic potential in treating various diseases, including lung cancer. Several studies have reported the pro-apoptotic effects of TQ in lung carcinoma models, highlighting its promise as an anti-cancer agent (23).

In vivo studies by Gajendra et al. (2022) demonstrated that TQ effectively attenuated lung carcinogenesis induced by Benzo(a)pyrene (B(a)P) in Swiss Albino mice. Administration of TQ at a dose of 20 mg resulted in improved body weight, reduction in lung weight, and a decreased mortality rate of 10% in B(a)P-induced lung cancer models (24). B(a)P-diol-epoxide (BPDE), an active metabolite of B(a)P, is a potent carcinogen capable of forming DNA adducts and causing chromosomal abnormalities, underscoring the severity of its carcinogenicity.

Further advancements in TQ delivery were reported by Khan et al. (2022), who fabricated pH-sensitive liposomes encapsulating TQ (TQPSL) for targeted therapy in B(a)P-exposed mice. The pH-responsive liposomes were designed to prevent premature drug release under physiological pH, enabling efficient TQ delivery in the acidic tumor microenvironment. Mice treated with TQPSL after 6-8 weeks of B(a)P exposure exhibited significant reductions in biochemical markers associated with lung cancer progression, including A, γ-GT, AHH, ADA, 5’-NT, and LDH. Importantly, pretreatment with TQPSL markedly inhibited lung cancer cell proliferation (25).

At the cellular level, TQ exerts its anti-cancer effects by modulating multiple signaling pathways. Yang et al. (2015) demonstrated that TQ suppresses proliferation, invasion, and migration of A549 lung cancer cells through inhibition of the ERK1/2 signaling pathway. Specifically, TQ reduced the phosphorylation of ERK1/2, leading to decreased expression of proliferative markers such as PCNA, cyclin D1, and matrix metalloproteinases MMP2 and MMP9, as well as downregulation of P16 and gelatinase activity (26). Additional studies using phytosomal formulations of TQ showed cell cycle arrest in the G2/M and pre-G1 phases and significant elevation in reactive oxygen species (ROS) levels, implicating oxidative stress as a mechanism of apoptosis induction (27).TQ also targets microtubule dynamics, a critical factor in maintaining cellular structure and mitosis. Direct interaction of TQ with tubulin polymers leads to their depolymerization, causing cell cycle arrest in the G2/M phase (28). Furthermore, TQ has been shown to reduce levels of ENA-78 and GRO cytokines, which are involved in angiogenesis and neovascularization, thereby contributing to its anti-angiogenic effects. Annexin-V staining revealed a high percentage (73.5%) of apoptosis in treated cells (29). Another notable mechanism involves TQ binding to G-quadruplex (G4) DNA structures at telomeric ends, resulting in cell cycle arrest at the G1 phase and increased cellular senescence (30).To enhance the bioavailability and efficacy of TQ, various nanoparticle-based delivery systems have been developed. PEGylated PLGA nanoparticles conjugated with transferrin

(TF-TQ-Np) demonstrated improved anti-cancer activity by upregulating tumor-suppressive microRNAs, including miR-34a and miR-16, which inhibit the anti-apoptotic protein Bcl-2, thereby promoting apoptosis in A549 cells (31). In a similar vein, Upadhyay et al. (2021) reported that transferrin-conjugated nanoparticles co-delivering TQ and gefitinib effectively overcame gefitinib resistance in A549 cells (32). Additionally, phytophospholipid nanovesicles combined with scorpion venom enhanced TQ-induced apoptosis, increasing the expression of key pro-apoptotic genes such as p53, caspase-3, and Bax while modulating inflammatory markers like NF-κB and TNF-α, suggesting promising therapeutic potential in lung adenocarcinoma treatment (33).

Combination therapies involving TQ and other chemotherapeutic agents have demonstrated synergistic effects in inducing apoptosis. For instance, the combination of TQ with quercetin (TQ+Qu) in A549 cells resulted in 50.9% early apoptosis, surpassing the apoptosis rates induced by TQ (40.6%) or quercetin alone (33.3%). This combination also increased both early and late apoptosis phases and downregulated pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression, highlighting enhanced cytotoxicity (34). Similarly, TQ combined with cisplatin (CDDP) significantly reduced tumor volume and weight in mouse xenograft models in a dose-dependent manner. This combination also modulated NF-κB signaling pathways and inhibited Polo-like kinases, key regulators of mitotic progression (35).

Moreover, Dera et al. (2020) observed that the combination of indirubin-3-monoxime (I3M) and TQ decreased cell viability, increased apoptosis, and caused G0 phase cell cycle arrest in vitro. This combination also impaired metastatic potential and tumor growth in vivo, downregulating oncogenic markers such as p-AKT, p-mTOR, caspase-3, p53, and NF-κB, further supporting the synergistic anti-carcinogenic effects (36). Similarly, the TQ and resveratrol combination reduced cellular viability in A549 cells, accompanied by increased expression of apoptotic genes including Bax, Fas, FasL, p53, and caspases 3, 8, and 9. This combination also elevated levels of p53, p27, and p21 and disrupted mitochondrial membrane integrity, potentiating apoptotic pathways (37)

Chapter: 3 MATERIALS AND METHODS:-

3.1 Cell Culture:

The primary goal of this procedure was to establish, maintain, and propagate a healthy population of A549 cells (a non-small cell lung carcinoma line) under optimal in vitro conditions to serve as the biological model for evaluating the apoptotic response to thymoquinone.

Materials and Reagents

- A549 cell line
- Dulbecco's Modified Eagle Medium (DMEM), high glucose formulation
- Fetal Bovine Serum (FBS), heat-inactivated
- Penicillin-Streptomycin solution (100×)
- 0.25% Trypsin-EDTA
- Phosphate Buffered Saline (PBS), pH-adjusted to 7.4
- Sterile T-flasks (T-25) and T-75)
- Dimethyl sulfoxide (DMSO)
- CO_2 Incubator (37°C, 5% CO_2 atmosphere)
- Class II Biosafety Cabinet
- Inverted Phase Contrast Microscope

Reagent Preparations

Complete Culture Medium

- Combine DMEM with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin.
- Mix gently and store at 4°C. Pre-warm to 37°C before use.

Phosphate Buffered Saline (PBS) – 1 L

- Sodium chloride: 8.0 g
- Potassium chloride: 0.2 g
- Disodium hydrogen phosphate: 1.44 g
- Potassium dihydrogen phosphate: 0.24 g
- Adjust final pH to 7.4, sterilize by autoclaving.

Protocol

Thawing of A549 Cells

- Rapidly thaw cryopreserved vials in a 37°C water bath (1–2 minutes).
- Decontaminate the outer surface with 70% ethanol before placing in the biosafety hood.
- Gently transfer the thawed contents into 5 mL of warm complete medium.
- Centrifuge at 1000 rpm for 5 minutes to remove DMSO.
- Discard the supernatant and resuspend cells in fresh medium.
- Transfer the suspension into a sterile T-25 flask and incubate under standard culture conditions.

Routine Culture Maintenance

- Inspect cells daily for morphology and confluency.
- Replace old medium every 48–72 hours or sooner if media color change suggests nutrient depletion.
- To replace the medium:
    - Aspirate spent media without disturbing the monolayer.
    - Add pre-warmed complete medium gently to avoid detaching adherent cells.

Sub-Culturing of Cells

- Conduct passaging once the monolayer reaches approximately 70–80% confluency.
- Remove medium and rinse gently with sterile PBS.
- Add 1 mL of Trypsin-EDTA to detach cells and incubate at 37°C for 2–3 minutes.
- Observe under microscope until detachment is complete.
- Add 4 mL fresh complete medium to neutralize the enzyme.
- Transfer to a tube, spin down at 1000 rpm for 5 min.
- Discard the supernatant and re-suspend the pellet in 5 mL of growth media.
- Re-seed into new flasks at a ratio of 1:4 or 1:5 as needed.

3.2 MTT Assay

The aim of the MTT assay was to evaluate the cytotoxic and anti-proliferative effects of Thymoquinone on A549 human lung carcinoma cells by assessing cell metabolic activity as an indirect indicator of cell viability following treatment.

The MTT assay is a colorimetric technique used to assess the viability and proliferation of cells based on their metabolic activity. The assay employs 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow-colored, water-soluble tetrazolium salt.

Viable cells possess active mitochondrial dehydrogenase enzymes that reduce MTT into insoluble purple formazan crystals. This reduction occurs only in metabolically active (living) cells, while dead or non-proliferating cells cannot perform this transformation. The formazan is then dissolved using an organic solvent such as dimethyl sulfoxide (DMSO) to yield a colored solution, the intensity of which is directly proportional to the number of viable cells. The absorbance is quantitatively measured using a spectrophotometer or microplate reader at a wavelength of 570 nm, with a reference filter often set at 630–690 nm to subtract background.

This assay provides a reliable estimation of cell survival, cytotoxic effects of drugs, and potential anti-cancer activity of compounds such as thymoquinone.

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Dulbecco's Modified Eagle Medium (DMEM), with 10% FBS and 1% Pen-Strep
- Thymoquinone (TQ)
- MTT reagent (Sigma-Aldrich or equivalent)
- Dimethyl sulfoxide (DMSO)
- 96-well flat-bottom tissue culture plates
- CO_2 incubator
- Inverted phase contrast microscope
- Microplate reader or ELISA plate reader (570 nm)

Reagent Preparation

MTT Stock Solution (5 mg/mL)

- Dissolve 5 mg of MTT in 1 mL of sterile PBS (filter-sterilized using a 0.22 µm filter).
- Store in the dark at 4°C for up to 2 weeks.

DMSO (Formazan Solubilization Solution)

- Use analytical grade DMSO directly.
- Store at room temperature, protect from moisture.

Protocol

Pre-treatment Setup

- Seed A549 cells into a 96-well plate at a density of 5×10^3 to 1×10^4 cells per well in 100 µL complete DMEM.
- Incubate the plate overnight at 37°C in a humidified incubator with 5% CO_2 to allow cell attachment.

Treatment with Thymoquinone

- Prepare different concentrations of thymoquinone (e.g.0.5,1,1.5,2,2.5,3,3.5,4,4.5,5,7,8,9 10 ug/ml) in complete medium.
- Replace the old medium in each well with 100 µL of TQ-containing medium.
- Include untreated control wells (only medium) and vehicle control (e.g., DMSO without TQ).
- Incubate for 24, 48, and/or 72 hours depending on experimental design.

MTT Addition

- After the treatment period, add 10 µL of MTT stock solution (5 mg/mL) into each well to achieve a final concentration of 0.5 mg/mL.
- Incubate the plate for 3–4 hours at 37°C. During this time, purple formazan crystals will form in viable cells.

Solubilization and Reading

- Add 100 µL of DMSO to each well to dissolve the formazan crystals.
- Shake the plate gently for 10–15 minutes at room temperature in the dark to ensure complete solubilization.

- Measure the absorbance at 570 nm using a microplate reader.
- A reference wavelength of 630 nm may be used to subtract background absorbance.

3.3 Cell Cycle Analysis by Flow Cytometry

The objective of this experiment was to determine the effect of Thymoquinone (TQ) treatment on the distribution of A549 lung carcinoma cells across different phases of the cell cycle (G_o/G_1, S, and G_2/M phases) using propidium iodide (PI) staining followed by flow cytometric analysis.

Cell cycle analysis using flow cytometry allows for the quantification of DNA content in individual cells, which reflects their progression through the cell cycle. In this assay, cells are stained with propidium iodide (PI), a fluorescent intercalating agent that binds stoichiometrically to DNA. Since DNA content doubles as a cell progresses from G_1 to G_2/M phase, PI fluorescence intensity directly correlates with the amount of DNA present.

The protocol involves:

- Fixing cells in ethanol to permeabilize the membrane.
- Treating with RNase A to degrade RNA (which also binds PI and would otherwise confound results).
- Staining DNA with PI.

Using flow cytometry, cells in G_o/G_1 phase (2N DNA), S phase (synthesizing DNA), and G_2/M phase (4N DNA) are distinguished based on their fluorescence intensity. The percentage of cells in each phase provides insight into cell cycle arrest or progression induced by TQ treatment.

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Complete DMEM with 10% FBS and 1% Pen-Strep
- Thymoquinone (TQ)
- 1X Phosphate Buffered Saline (PBS)
- Ice-cold 70% ethanol
- RNase A (10 mg/mL)

- Propidium iodide (PI, 1 mg/mL stock)
- FACS tubes
- Flow cytometer (with appropriate red fluorescence detector, e.g., FL2 or FL3 channel)
- Microcentrifuge and cooling centrifuge

Reagent Recipes

- 70% Ethanol: Mix 70 mL absolute ethanol with 30 mL sterile distilled water; chill at −20°C.
- PI Staining Solution (for 1 mL):
    - PI: final concentration of 50 µg/mL
    - RNase A: final concentration of 100 µg/mL
    - Dilute both in 1X PBS, prepare fresh or store aliquots at −20°C.

Protocol

Cell Treatment and Harvesting

- Seed A549 cells in 6-well plates and allow them to adhere overnight.
- Treat cells with desired concentrations of Thymoquinone for 24 or 48 hours.
- After treatment, collect both floating and adherent cells by trypsinization and pooling.

Fixation

- Wash the cell pellet once with ice-cold PBS.
- Resuspend cells in 0.5 mL PBS.
- Add 4.5 mL of pre-chilled 70% ethanol dropwise while vortexing gently.
- Incubate at −20°C for at least 2 hours or overnight for proper fixation.

RNA Removal and DNA Staining

- Centrifuge fixed cells at 300 × g for 5 minutes and discard the ethanol.
- Wash the pellet twice with PBS to remove residual ethanol.

- Resuspend cells in 500 µL PI staining solution (containing PI and RNase A).
- Incubate in the dark at 37°C for 30 minutes.

Flow Cytometry Analysis

- Transfer stained cells into FACS tubes.
- Filter through a nylon mesh or cell strainer to remove clumps (optional but recommended).
- Analyze samples using flow cytometry.
- Acquire at least 10,000 events per sample.
- Evaluate data using software like FlowJo or BD CellQuest to determine the percentage of cells in G_0/G_1, S, and G_2/M phases.

3.4 Annexin V/Propidium Iodide (PI) Assay

The aim of this experiment was to evaluate the extent of apoptosis induced by Thymoquinone (TQ) in A549 human lung cancer cells by using Annexin V-FITC/Propidium Iodide (PI) dual staining followed by flow cytometric analysis. This assay helps distinguish between live, early apoptotic, late apoptotic, and necrotic cells.Apoptosis, or programmed cell death, involves early changes in the plasma membrane, one of which is the externalization of phosphatidylserine (PS) from the inner to the outer leaflet. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, thus detecting cells undergoing early apoptosis.Propidium Iodide (PI) is a membrane-impermeable DNA-binding dye that only penetrates cells with compromised membrane integrity—marking late apoptotic or necrotic cells. When used together:

Cell Status	Annexin V-FITC	PI
Live cells	−	−
Early apoptosis	+	−
Late apoptosis	+	+
Necrosis	−	+

Table 1 :- Cell Status against staining with Annexin V-FITC and PI

By measuring fluorescence from both Annexin V-FITC (green) and PI (red), flow cytometry can classify and quantify cells in each category.

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Thymoquinone (TQ)
- Complete DMEM (with 10% FBS and 1% Pen-Strep)
- 1X Phosphate Buffered Saline (PBS)
- Annexin V-FITC Apoptosis Detection Kit (includes Binding Buffer, Annexin V-FITC, and PI)
- FACS tubes
- Trypsin-EDTA
- Flow cytometer (with FL1 and FL3/FL2 channels for FITC and PI respectively)

Reagent Recipes

- 1X Binding Buffer (if not provided in kit):
    - 10 mM HEPES, pH 7.4
    - 140 mM NaCl
    - 2.5 mM $CaCl_2$

Filter-sterilize and store at 4°C. Always prepare fresh before use if homemade.

Protocol

Cell Treatment and Harvesting

- Seed A549 cells in 6-well plates and incubate overnight for adherence.
- Treat cells with different concentrations of Thymoquinone for 24 or 48 hours.
- Collect both floating and adherent cells by trypsinization.
- Wash cells twice with cold 1X PBS by centrifuging at 300 × g for 5 minutes.

Staining for Apoptosis Detection

- Resuspend ~1×10^5 to 1×10^6 cells in 100 µL of 1X binding buffer.
- Add 5 µL Annexin V-FITC and 5 µL PI to the cell suspension.
- Gently vortex and incubate in the dark at room temperature for 15 minutes.
- After incubation, add 400 µL 1X binding buffer to each tube.

- Keep the tubes on ice and analyze samples within 1 hour by flow cytometry.

Flow Cytometric Analysis

- Set flow cytometer for FL1 (FITC) and FL2/FL3 (PI) detection.
- Collect data for at least 10,000 events per sample.
- Gate the cell population properly and use quadrant analysis to determine the percentage of cells in:
    - Live (Annexin−/PI−)
    - Early apoptotic (Annexin+/PI−)
    - Late apoptotic (Annexin+/PI+)
    - Necrotic (Annexin−/PI+)

3.5 WESTERN BLOTTING

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells.

The technique uses three elements to accomplish this task:

(1) separation by size

(2) transfer to a solid support

(3) marking target protein using a proper primary and secondary antibody to visualize.

A synthetic or animal-derived antibody (known as the primary antibody) is created that recognizes and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein.Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

PROCEDURE :

Protein Isolation :

RIPA Buffer recipe

Components	Volume
1M NaCl	750µl
Triton X-100	50µl
10% SDS	500µl
0.5M EDTA	100µl
1.5M Tris-HCL (pH = 8.0)	170µl
10% glycerol	500µl
Milli-Q water	2930µl

Table 2 :- RIPA Buffer composition

For protein isolation or protein purification there are the following steps –

1) ➢ Firstly, appropriate cells are cultured in DMEM media buffer and store in an Eppendorf.
2) ➢ Then it is centrifuged at 3000 rpm for 3 minutes in 4 degree C. The pellet should be collected and resuspend in a Eppendorf with DMEM medium buffer.
3) ➢ Centrifuge at 3000 rpm for 3 minutes in 4 degree C.
4) ➢ Pipette out the supernatant and add PBS wash buffer to the collected pellet.
5) ➢ Then centrifugation has to be done at 3000 rpm for 3 minutes in 4 degree C .
6) ➢ Pipette out the wash buffer (supernatant) PBS .
7) ➢ The pellet is collected in an Eppendorf and mixed with RIPA buffer for lysis of the cell and extraction of protein
8) ➢ Store in ice box for 15 minutes at 4 degree C.
9) ➢ The RIPA buffer containing the pellet is again resuspended and again store in ice box for 15 minutes at 4 degree C.
10) ➢ Repeat the process of resuspension and storing for three times.
11) ➢ Finally, centrifugation has to be done at 13000 rpm for 25 minutes at 4 degree C.
12) ➢ Here, the pellet is the cell debris which has to be removed . The supernatant is the extracted protein. Obtain the protein for the next step – Protein Estimation.

2) Protein Estimation :

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and 15 phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents Required :

a) BSA Protein Assay Reagent A : 2% sodium carbonate in 0.1 N sodium hydroxide.

b) BSA Protein Assay Reagent B : 0.5% copper sulphate (CuSO4.5H2O) in 1% potassium sodium tartarate. Prepare fresh by mixing stock solutions.

c) BSA Kit : BSA 1 mg/ml , BSA 3 mg/ml , BSA 5 mg/ml.

d) Distilled water

e) Protein 1 and Protein 2.

Procedure:

➢ Mix (100×n) µl [n = no. of wells] of Reagent A and (2×n) µl of Reagent B. So for 6 wells there should be (6×100) µl i.e. 600 µl of reagent A and (2×6) µl i.e. 12 µl.

➢ Take a well plate and mark 6 wells in a row as Blank, sample 1, sample 2, sample 3, Unknown 1, Unknown 2.

	Blank	Sample 1	Sample 2	Sample 3	Unknown 1	Unknown 2
Reagent A+B	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Distilled water	15 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl
BSA / Protein		2.5 µl (1mg/ml) BSA	2.5 µl (3mg/ml) BSA	2.5 µl (5mg/ml) BSA	2.5 µl Protein 1	2.5 µl Protein 2

Table 3:- BCA protein estimation assay

- Incubate the well plate in a dark environment for at least 30 minutes.
- Check the absorbance in Multiscan Go.

Separation of protein by SDS-PAGE:-

For a 5 ml stacking gel	
H_2O	2.975 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

Table 4:- Stacking gel recipe

For a 10ml separating gel

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µ l	100µ l	100µ l	100µ l	100µ l
TEMED	10µ l	10µ l	10µ l	10µ l	10µ l

Note: AP and TEMED must be added right before each use.

Table 5:- Separating gel recipe

5X SDS Loading Sample Buffer (10 mL)

250 mM Tris HCl, pH 6.8 – 2.5 mL from 1M stock
10% SDS – 1 g
30% Glycerol – 3 mL
7.5% β-mercaptolethanol – 0.75 mL
0.05% bromophenol blue – 0.5 mL from 1% stock

Table 6:- Sample loading buffer

SDS-PAGE Electrophoresis Running Buffer (1X) [1X: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3]- 1lt.

3.03 g Tris base (MW 121.1)
14.40 g Glycine (MW 75.07)
1 g SDS

No need to adjust pH

Table 7:- Electrophoresis Running Buffer

Sample Preparation Buffers

1 M Tris-HCl, pH 7.6 (100 ml)

Tris base	12.11 g
Deionized H_2O (diH_2O)	80 ml
Adjust pH to 7.6 with HCl.	
diH_2O	to 100 ml

0.5 M Tris-HCl, pH 6.8 (100 ml)
(catalog #1610799)

Tris base	6.06 g
diH_2O	~60 ml
Adjust to pH 6.8 with HCl.	
diH_2O	to 100 ml
Store at 4°C.	

10% SDS (10 ml)
(catalog #1610416)

SDS	1.00 g
diH_2O	to 10 ml

1.0% Bromophenol Blue (10 ml)
(catalog #1610404)

Bromophenol blue	100 mg
diH_2O	to 10 ml

Table 8:- Sample preparation Buffers

Gel Casting Reagents

Acrylamide/Bis (30% T, 2.67% C)

Acrylamide (29.2 g/100 ml)	87.60 g
N'N'-bis-methylene-acrylamide	2.40 g
diH_2O	to 300 ml
Filter and store at 4°C in the dark (30 days).	

Premade alternatives:
Catalog #161-0125, 37.5:1 acrylamide/bis powder
Catalog #161-0158, 30% acrylamide/bis solution

1.5 M Tris-HCl, pH 8.8 (150 ml)
(catalog #1610798, 1 L)

Tris base (18.15 g/100 ml)	27.23 g
diH_2O	80 ml
Adjust to pH 8.8 with 6 N HCl.	
diH_2O	to 150 ml
Store at 4°C.	

0.5 M Tris-HCl, pH 6.8
(catalog #1610799, 1 L)

Tris base	6.00 g
diH_2O	60 ml
Adjust to pH 6.87 with 6 N HCl.	
diH_2O	to 100 ml
Store at 4°C.	

10% (w/v) SDS (100 ml)
(catalog #1610416)

SDS	10.00 g
diH_2O	90 ml
Dissolve with gentle stirring	
diH_2O	to 100 ml

10% (w/v) APS (fresh daily)
(catalog #1610700)

Ammonium persulfate	0.10 g
diH_2O	1 ml

Water-Saturated *n*-Butanol

n-Butanol	50 ml
diH_2O	5 ml
Combine in a bottle and shake. Use the top phase	
Store at room temperature.	

Table 9:- Gel casting Reagents

Gel Casting :

❖ Assembling the components that you will need for casting the gel: a thick glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.

❖ Placing the green casting frame on the bench with the green “feet”resting firmly against the bench and the clamps open (perpendicular to the frame) and facing the working individual.

❖ Placing the two gel plates in the frame(if only one gel is required then using a dummy plate is appropiate) . Insert the thicker spacer plate with the “UP” arrows up and the spacers facing toward the individual into the casting frame (the BioRad logo should be facing you). Inserting the short glass plate in the front of the casting frame. There should be a space between the plates.

❖ Securing the plates in the casting frame by pushing the two gates of the frame out to the sides.

❖ Clamping the casting frame with glass plates into the casting stand, with the gates of the casting frame facing you.

❖ Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of deionized water into the gap between the plates. If the glass plates hold water and don’t leak, it is ready to make the gels. Pour the water out by holding the entire cast- ing platform over a liquid waste container or sink. Use paper towels or tissues to absorb any residual water. If the gel leaks, disassemble the frame, dry the plates and re assemble.

❖ Using a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height just above the green clamps on the gel casting frame.

❖ Using a transfer pipet, add butanol so that it gently flows across the surface of the polyacrylamide mixture. The water layer ensures that the polyacrylamide gel will have a level surface once it polymerizes.

❖ When polymerization is complete, remove the butanol from the top of the resolving gel by tilting the gel to the side and using a tissue.

❖ Using a transfer pipette to pipette the stacking gel on top of the resolving gel between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.

❖Carefully, but quickly, lower the comb into position, being careful not to introduce air bubbles. (The Bio Rad logo on the comb should be facing you.) Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.

❖ Carefully remove the comb from the spacer gel.

❖ Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward. Place a second gel cassette or a buffer dam against the gasket in the other side of the electrode assembly.

❖ Clamp the green clamps on the sides of the electrode assembly .

❖ Lower the chamber into the electrophoresis tank.

❖ Fill the space between the two gels with Tris-glycine running buffer. This forms the upper chamber for electrophoresis.

❖ Add Tris-glycine running buffer (electrophoresis buffer) to the outer (lower) chamber until the level is high enough to cover the platinum wire in the electrode assembly. 18 Gel Loading.

❖ Prepare mixture of protein and loading dye . 20 µl of protein and 5 µl of loading dye has to be prepared for running in a single well. For preparing loading protein for 4 wells we have to prepare 20×4 = 80 µl of protein and 5×4 µl loading dye mixed properly

❖ Set the micropipette at 25 µl and take from the prepared protein-loading dye mixture and put it in their respective well

❖ Load samples slowly and allow the samples to settle evenly on the bottom of the well.

Running Gel :

❖ Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).

❖ Turn on the power supply. Run the gel at 90V until the sample protein reaches the bottom of the stacking gel and then run the gel at a constant voltage of 120 V resolving gel. Run the gel until the blue dye front nearly reaches the bottom of the gel.

Blotting :

In order to make protein accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or poly viniledene di fluoride (PVDF). Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol. The transfer procedure has been performed for the quantitative recovery of proteins from SDS-containing polyacrylamide gels.

Procedure:-

❖ Cut the gel with a gel cutter from the gel plate and cut into a size for transferring the bands to the membrane.

❖ Take the PDVF membrane and wet the PDVF membrane in methanol for activation of the membrane.

❖ Wet the sponge and filter papers

❖ Make a sandwich .

❖ Carefully place the cathode plate onto the stack. Press to engage the latches with the guide posts without disturbing the gel/nitrocellulose stack.

❖ Added transfer buffer to the apparatus, and ensure that the sandwich was covered with the buffer.

❖ All components in contact with the slab gel were prewetted in the electrode solution (Transfer Buffer) composed of 20 mM Tris base, 150 mM glycine, and 20% methanol.

❖ This solution should be degassed briefly under vacuum before use. Here, the method for transferring proteins is called electroblotting, where electric current was used to pull proteins from the gel into membranes within the machine Trans-Blot Turbo Transfer System by BIO RAD.

Binding of antibody to nitrocellulose-immobilized proteins:

❖ Blocking is a very important step of western blotting, as it prevents antibodies from binding to the membrane nonspecifically.

❖ Immediately following transfer, the nitrocellulose sheet is immersed in a mild blocking solution containing non fat dried milk powder in Tris Buffer Saline (TBS) along with a detergent, Tween 20 (together called TBST) and incubated at 40°C for 1hr on a rocking platform to prevent the non-specific interactions between the membrane and the antibody used for the detection of the target protein.

❖ The nitrocellulose was then washed with rocking for 10 min in Tris-saline for three times. The sheet is immersed in Primary antibody solution on a rocking platform overnight at 4°C.

❖ Then next day the sheet was kept in room temperature for 1 hour on rocking condition. then sheet was again washed with rocking for 10 min in Tris-saline for three times.

1x Transfer buffer (200ml)	
Components	**Volume**
10x Transfer buffer	20 ml
Water	140 ml
Methanol	20 ml

Table 10:- Transfer Buffer Recipe

10x TBS (pH = 7.5 to 8.0)	
Components	**Volume**
Tris-Base	60.5 gm
NaCl	87.6 gm
Water	800 ml
Adjust pH using HCl and then make volume to 1000ml using water.	

Table 11:- TBS recipe

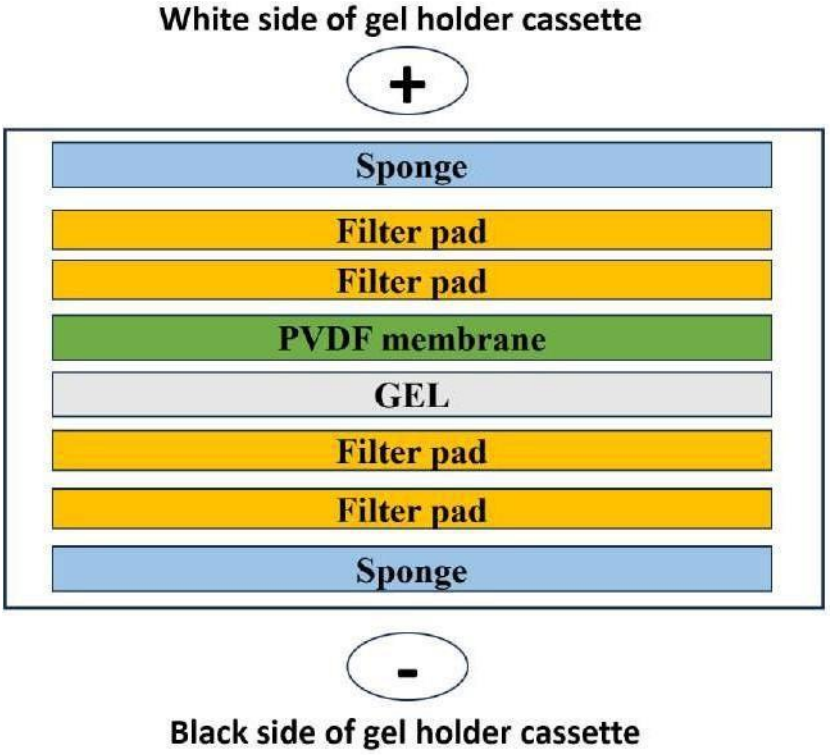


Fig 1:- **Wet transfer setup for Western blotting**

❖ After that Secondary antibody was added to the sheet and placed on a rocking platform at room temperature for 2 hr.

❖ Washing is very important as it minimized background and removes unbound antibody. However, the membrane should not be left to wash for a really long time, as it can also reduce the signal.

❖ The nitrocellulose sheet was then washed with rocking for 10 min in Tris-saline for three times.

❖ After that, the developing solution (containing Luminol and p-Coumaric acid) was applied on the nitrocellulose sheet, and incubated in dark room on rocking condition for 2 to 3 mins, which will form dipharmazone, a blue coloured complex; catalysed by the enzyme alkaline phosphatase (ALP), which was present in the Secondary antibody, i.e.; wherever Secondary antibody is present, blue coloured band will develop there.

❖ The sheet was then scanned in Chemi-Doc XR+ by BIO RAD and the images are taken and saved.

Chapter 4 : Results and Discussion

4.1 A549 cell line

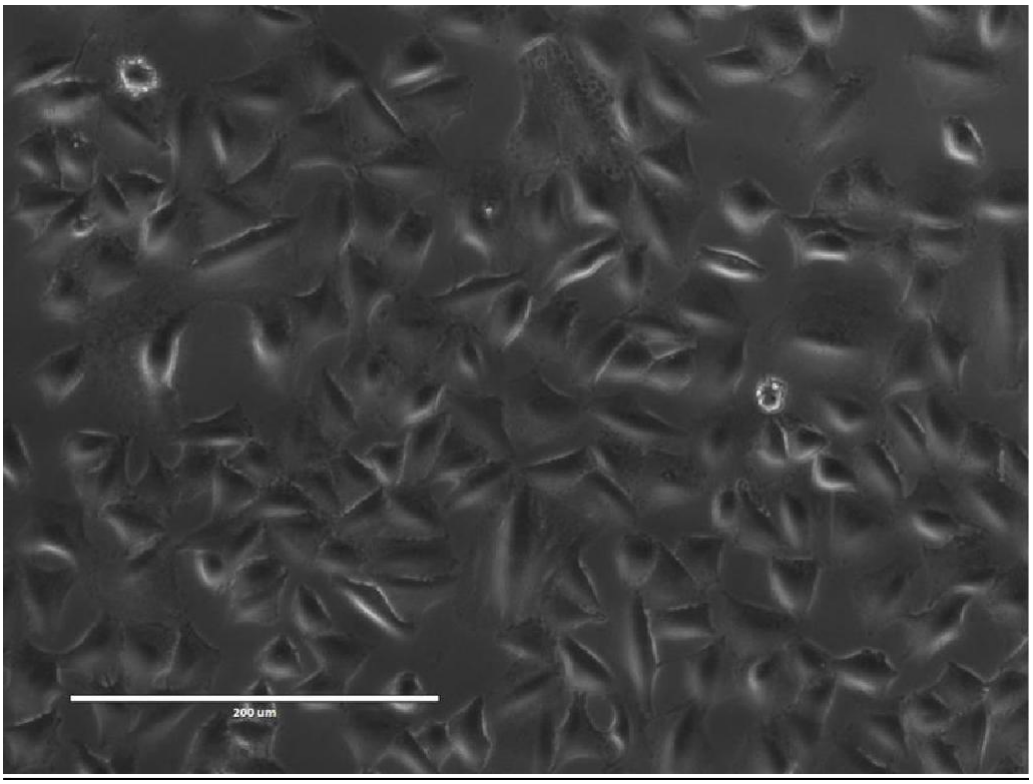
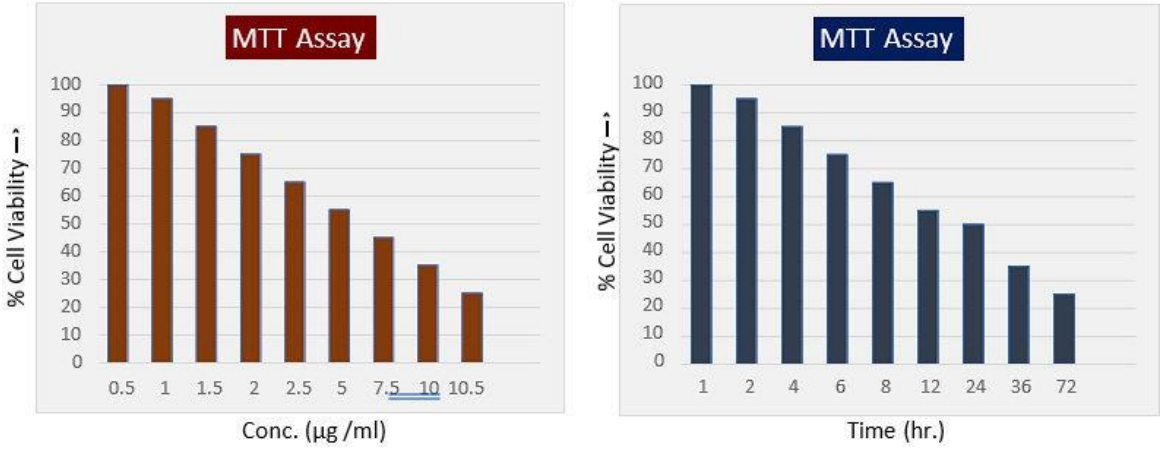


Fig 2:- A549 Cell line

A549 cells are adenocarcinomic human alveolar basal epithelial cells, and constitute a cell line that was first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male. The cells are used as models for the study of lung cancer and the development of drug therapies against it.

Characteristics: A549 cells, as found in the lung tissue of their origin, are squamous and responsible for the diffusion of some substances, such as water and electrolytes, across alveoli. If A549 cells are cultured in vitro, they grow as a monolayer; adherent or attaching to the culture flask. The cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids, which are important to maintain membrane phospholipids. A549 cells are widely used as a type II pulmonary epithelial cell model for drug metabolism and as a transfection host. When grown for a sufficiently long time in cell culture, A549 cells may begin to differentiate, as signaled by the presence of multilamellar bodies.

4.2 A549 cell viability analysis on the application of TQ through MTT Assay:-

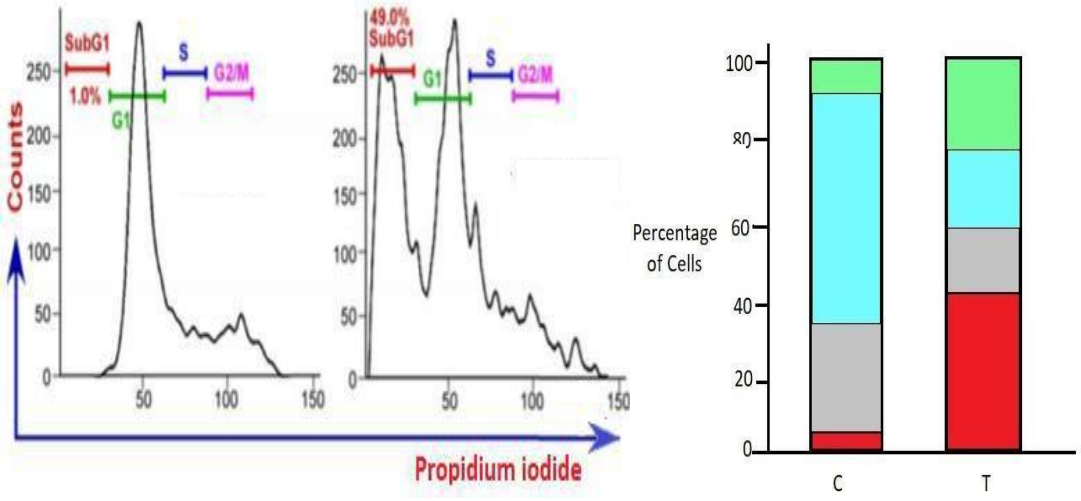


(a) **(b)**

Figure 3 - Analysis of percentage of cell survival by MTT assay on A549 cells on exposure to TQ (a) in increasing TQ concentrations (0-10.5/ml) of TQ after 24 h of incubation (b) at different time points (1,2, 4, 6, 8,12, 24, 36, 48 and 72 h), in an increasing manner.

As shown in Fig., exposure of A549 cells (a) in increasing TQ concentrations (0-10µg/ml) of TQ for 24 h, decreased the percentage of cellular viability in a concentration/dose-dependent manner. The IC50 concentrations (producing half maximal inhibition) at 24 h were between 2.5 and 5µg/ml of TQ for all cells, and the IC50 was 5µg/ml of TQ. In Fig. (b), at different (increasing) time points (1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h), reduced the percentage of cell survival in a time-dependent manner. It is also evident that the half maximal survival (50%) of cells occurs at 24 h.

4.3 Cell cycle analysis:-

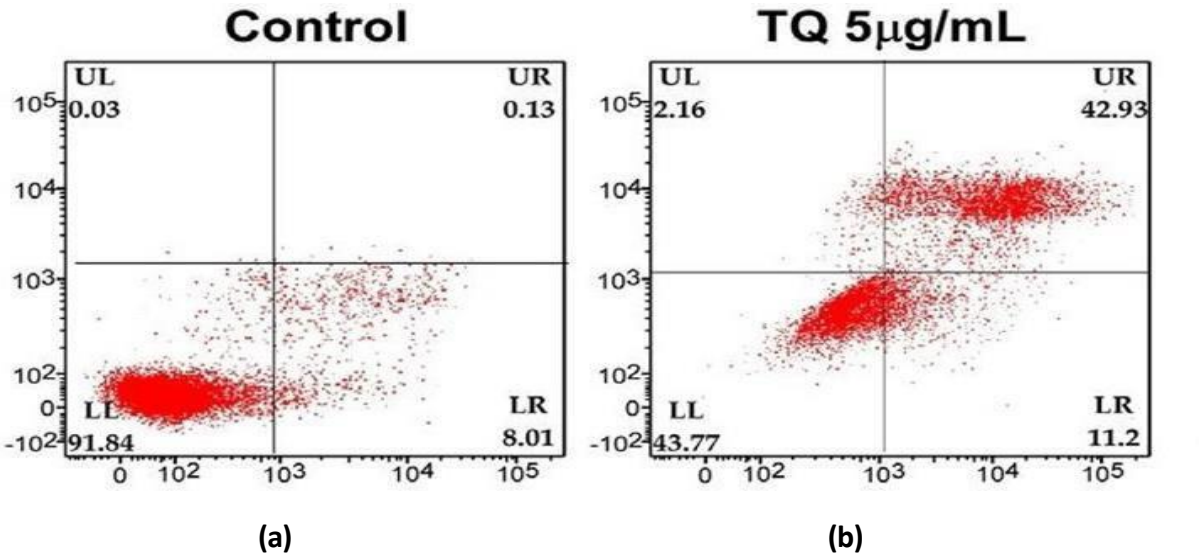


(a) 1: Untreated **(a) 2: TQ (5µg/ml).** **(b)**

Fig 4: Analysis of percentage of cell death or cellular toxicity by flow-cytometric cell cycle analysis on A549 cells, (a) 1: one control set up (without any treatment), (a) 2: along with dose of TQ (5µg/ml). (b) Graphical representation of Cell cycle analysis.

Cell cycle analysis was conducted to determine the effect of thymoquinone (TQ) at 5 µg/mL on A549 lung cancer cells. The data showed a significant increase in the sub-G1 population, indicating DNA fragmentation and apoptosis. This suggests that TQ induces apoptotic cell death rather than arresting cells at a specific checkpoint. A slight decrease in the S-phase population was also observed, implying reduced DNA synthesis and inhibition of cell proliferation. Minor changes in the G2/M phase suggest that TQ does not strongly interfere with mitotic progression. The prominent accumulation of cells in the sub-G1 phase aligns with previous findings that TQ triggers apoptotic pathways. These results support the notion that TQ at 5 µg/mL disrupts cell cycle progression and promotes apoptosis in A549 cells. Thus, TQ demonstrates potent anti-proliferative and pro-apoptotic effects, reinforcing its potential as an effective anticancer agent for lung cancer therapy.

4.4 Determining cellular apoptosis using Annexin V/ PI Assay:



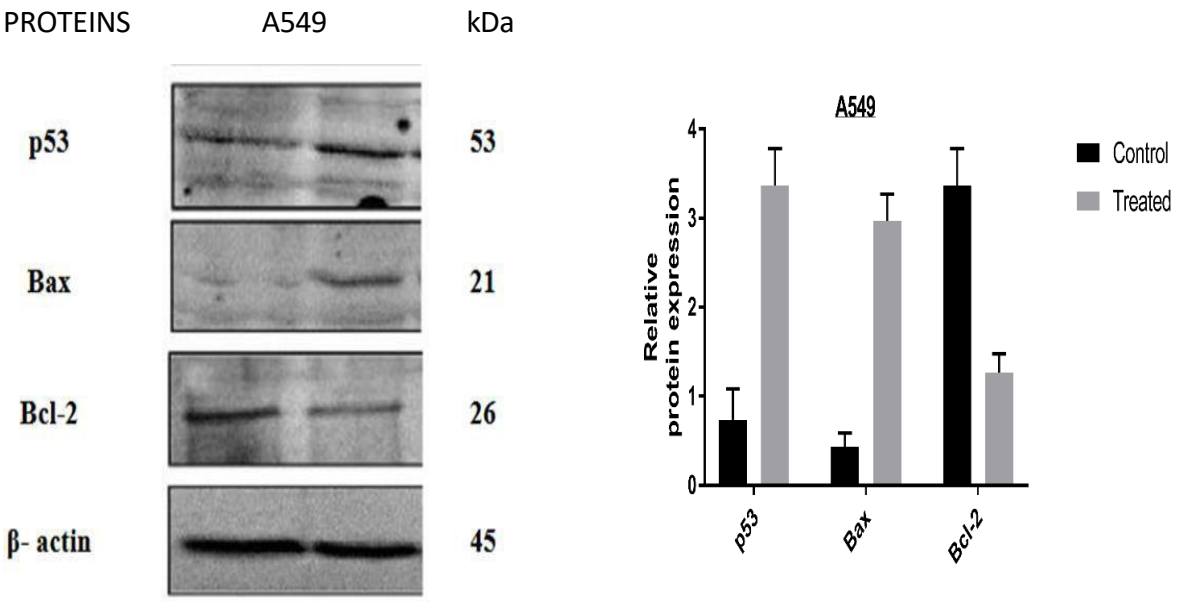
(a) **(b)**

Fig 5:- The representation of apoptotic and necrotic populations of cells treated with indicated doses of TQ in A549 cells using Annexin V/PI in flow cytometry. (a) One control set up (without any treatment), (b) along with a dose of TQ (5μg/ml).

A549 cells were labelled with PI and Annexin V-FITC and analysed on a flow cytometer Dual parameter – quadratic dot plot of Annexin V (y-axis) versus PI fluorescence (x-axis) has been shown in logarithmic fluorescence intensity. Annexin v is a marker of apoptosis, while PI is a marker for necrosis.

The treatment of A549 cells with TQ increased the rate of apoptotic cell death. This finding thereby established the fact that the TQ showed apoptotic cell death in lung cancer cells at dose (5μg/ml) as compared to the control set up (with no treatment). Quantitative evaluation of apoptosis by annexin V/PI test indicated that the ratios of apoptotic cells significantly increased after TQ treatment. Using flow cytometry, the annexin V–FITC test confirmed the apoptotic potential of TQ against A549 cancerous cells.Cells that were propidium iodide (PI) negative and Annexin V negative (4th quadrant) are considered healthy, cells, PI negative and Annexin V positive cells (1st quadrant) are considered apoptotic, and cells that are positive to both PI and Annexin V (2nd quadrant) considered necrotic .

4.5 **Analysing the expression pattern of protein through Western Blot:**



(a) (b)

Fig. 5: Western blot analysis showing expression levels of p53, BAX and BCL-2 proteins in A549 cells, both in – (a) control set up and after TQ treatment with β- actin as the loading control.(b) Graphical representation of the same.

p53 and Bax are important pro-apoptotic regulatory proteins, while Bcl-2 functions as an anti-apoptotic protein that inhibits cell death. Assessing their expression is vital to understanding the apoptotic potential of therapeutic agents. Western blotting is a standard technique used to measure protein expression. In this study, we aimed to explore the molecular mechanism behind the anti-migratory effect of thymoquinone (TQ) on A549 lung cancer cells, with a focus on p53, a known tumor suppressor gene. TQ has been reported to upregulate p53, which can further activate downstream targets such as miR-34a, known to promote apoptosis. We performed Western blot analysis to examine the expression levels of p53, Bax, and Bcl-2 in TQ-treated and untreated A549 cells. The results showed a clear increase in p53 and Bax expression, along with a notable decrease in Bcl-2 levels upon TQ treatment, indicating activation of the intrinsic apoptotic pathway. These findings were further supported by immunofluorescence imaging. β-actin was used as a housekeeping protein and loading control to validate the consistency of protein loading. Altogether, the results suggest that TQ promotes apoptosis in A549 cells by upregulating p53 and Bax, while downregulating Bcl-2, supporting its potential as an anti-cancer agent.

Chapter 5 : -*Conclusion*

Determining the cellular viability with MTT assay as shown in Fig.1 revealed decreased cellular viability with increasing TQ concentrations (0-10ug/ml) for 24hrs. (a)The half maximal inhibitory concentration , IC50 value of the treated cells was between 2.5 ug/ml and 5ug/ml. (b) A reduction in the percentage of treated cell was observed at various time points(1, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 hrs). The IC50 value was dominant at 24hrs.To find out cellular apoptosis on inducing our NSLC cell line, A549 with TQ we performed flow cytometric analysis. Our treated and untreated A549 cell line was tagged using FITC-Annexin V propidium iodide (PI) and FITC-Annexin V and was then analyzed on a flow cytometer. The depiction of logarithmic fluorescence intensity was shown by the dual parameter – quadratic dot plot of FITC fluorescence (x-axis) versus PI fluorescence (y-axis). Annexin v is a marker of apoptosis, while PI is a marker for necrosis. Treating the A549 cells with thymoquinone resulted in increase in the apoptosis rate. Thereby, this finding determinates the fact that the TQ showed apoptosis in our cell line A549 at a dosage (5µg/ml, Fig.2) as compared to the control set up (with no treatment). Annexin V/PI test was used to quantitatively assess apoptosis. The results showed that following TQ treatment, the ratios of late-stage and early-stage apoptotic cells considerably rose from 8.01% and 1.13% (in the control setup, without treatment) to 11.2% and 42.89%, respectively. Hence, the apoptotic potential of TQ against the lung cancer cell line, A549 was proven using flow cytometry through the Annexin V-FITC test.Flow cytometric cell cycle phase distribution of the TQ treated A549 was performed to check the cytotoxic potential of TQ. We observed significant A549 cell death on treating the cells with TQ (5μg/ml). Prominent cellular cytotoxicity was observed at very low dosage 5μg/ml. Additional cell cycle analysis was performed, and TQ was found in inhibiting the molecules involved in S-phase following sub-G1 phase arrest occurred. To monitor the expression levels of the apoptotic gene p53, we presumed its participation in the investigation of TQ's antimigratory effects on A549 cells. It is a well-known fact that TQ induces cells to up-regulate their p53 levels. We performed Western blotting to check the expression pattern of p53, Bax and Bcl-2, keeping β-actin as the loading control or as housekeeping gene control.

protein on treating the A549 cells with TQ by keeping a control setup without treatment. This clearly indicated the induction apoptosis by p53 dependant pathways in the cells.The treatment of lung cancer cells with the bioactive compound derived from *N.sativa*, Thymoquinone (TQ) has shown to reduce cellular viability along with induced cell death in the A549cells. Our study has strongly reported the usage of TQ in exhibiting strong specificity towards the migration of cancer cell, with it being less toxic to our normal body cells. Our findings have also demonstrated the role of TQ in cell cycle arrest at the Sand sub G1 phase. Our current report has also focussed on delineating the pathways involved in the apoptosis of cancer cells. We found out that the underlying pathways involved in the apoptosis of A549 lung cancer cells, treated with TQ recruits the p53 dependent apoptotic pathways. The downregulation of the antiapoptotic gene Bcl2 and the upregulation of Bax were reported in our study. As a whole, we conclude that this lipophillic bioactive compound, TQ can be regarded as chemotherapeutic and chemopreventive substance in inducing cellular apoptotsis and viability of the NSCLC cell line, A549 cells.

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ABSTRACT :

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, accounting for approximately 12.4% of all cancer diagnoses and ranking as the most commonly diagnosed cancer globally. Non-Small Cell Lung Cancer (NSCLC) is the most prevalent subtype and presents significant therapeutic challenges due to the side effects, toxicity, and resistance associated with conventional treatments like chemotherapy and radiotherapy. Hence, the search for biocompatible, cost-effective alternatives with minimal side effects is a growing priority.

Thymoquinone (TQ), a bioactive compound derived from Nigella sativa (black seed), has been extensively studied for its anti-inflammatory, antioxidant, and antineoplastic

properties. The present in vitro study aimed to evaluate the anticancer potential of TQ on A549 human lung adenocarcinoma cells. A549 cells were treated with varying

concent TQ over different time durations, and their response was assessed using standard viability assays, protein expression analysis through western blotting, and flow cytometric quantification of apoptosis and necrosis via annexin V-FITC and propidium iodide staining.

The results demonstrated that TQ significantly reduced the viability of A549 cells in a dose- and time-dependent manner. Flow cytometry revealed increased apoptotic cell populations upon TQ treatment. Protein blotting data showed upregulation of p53 and Bax and downregulation of Bcl-2, indicating activation of intrinsic apoptotic pathways. Furthermore, enhanced expression of caspase-3 and caspase-9 confirmed caspase-dependent apoptosis.

These findings suggest that thymoquinone effectively promotes apoptosis in lung cancer cells and may serve as a promising therapeutic agent. However, further preclinical and clinical investigations are essential to validate its safety, pharmacokinetics, and efficacy in vivo.

Keywords

Thymoquinone (TQ); Nigella sativa; A549 lung cancer cells; Apoptosis; p53; Caspase-3; Bax/Bcl-2 ratio

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Chapter 1 :- INTRODUCTION

14 Lung cancer remains the leading cause of cancer-related deaths worldwide, both in terms of incidence and mortality. Despite continuous scientific advancements, the survival rate for lung cancer patients remains dismally low. In 2020 alone, approximately 1.8 million deaths were attributed to lung cancer, making up nearly 18% of all cancer deaths

globally (1–4). In the United States, around 200,000 new cases of lung cancer are diagnosed every

year. India accounts for 6.9% of new lung cancer cases and 5.9% of all cancers, with a significant concentration in metropolitan cities like Delhi, Chennai, and Bengaluru (5,6). Lung cancers are broadly categorized into two major types: Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer SCLC typically originates in the bronchi, affecting the tracheal region and rapidly progressing to deeper structures, including the lymph nodes (3). SCLC comprises two subtypes: small cell carcinoma and mixed small/large cell carcinoma, with small cell carcinoma being the more prevalent subtype, accounting for around 15% of lung cancers. The predominant cause of SCLC is tobacco smoking (3,4). Due to their rapid proliferation and early metastasis, SCLCs can be targeted for quicker treatment compared to NSCLCs (7).

In contrast, NSCLCs progress more slowly and are less aggressive than SCLCs. They account for a majority of lung cancer cases and are further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (8). Adenocarcinomas originate from the outer regions of the lung and often result in mucus production. Squamous cell carcinomas typically affect the central bronchi, and large cell carcinomas can develop in any lung region, often metastasizing rapidly. Despite their slower progression compared to SCLC, NSCLCs often have poor prognosis due to their aggressive metastatic behavior and resistance to conventional therapies.To understand the basis of lung cancer cell survival and the mechanism of action of therapies, it is crucial to understand apoptosis, the natural, regulated process of programmed cell death that eli The extrinsic pathway is triggered by extern tissue homeostasis. Apoptosis occurs through two primary signaling pathways: the extrinsic and the intrinsic pathways.

where death ligands bind to tumor necrosis factor (TNF) receptor family proteins on the cell surface, activating caspases, which are proteolytic enzymes that dismantle cellular structures (9). On the other hand, the intrinsic pathway is activated by

internal stressors like DNA damage or lack of growth factors, and is tightly regulated by pro-apoptotic proteins like Bax and anti-apoptotic proteins like Bcl-2. The shift in balance towards Bax leads to mitochondrial membrane permeabilization, cytochrome c release,

and ultimately caspase activation and cell death.Several proteins are integral to apoptosis, including the Bcl-2 family, p53, heat shock proteins, and amyloid-B peptide, with caspase-3 being the principal executioner caspase. It initiates the execution phase of apoptosis, leading to nuclear fragmentation, chromatin condensation, and apoptotic body formation (9).

[1]

Currently, chemotherapy remains the cornerstone of treatment for NSCLC, especially over the past three decades. The standard regimen for non-squamous NSCLC includes pemetrexed-platinum, along with drugs such as bevacizumab, paclitaxel, docetaxel, cisplatin, methotrexate, gefitinib, and porfimer (9). Combined therapies involving chemotherapy, radiotherapy, and prophylactic cranial irradiation have shown improved survival of up to 18–24 months in some patients (10). Neoadjuvant chemotherapy also shows promise in resectable NSCLC cases. However, these therapies often come with severe side effects, such as anemia, fatigue, infertility, infection, and organ toxicity—due to their non-selective targeting of both cancerous and normal cells. Similarly, while surgical techniques (like laser surgery, cryosurgery) can be effective, anesthesia complications and postoperative risks remain critical concerns (11). Despite global implementation, current treatments still leave significant gaps in efficacy and patient tolerance (12).

Thus, the search for safer, more targeted therapeutic options is ongoing. Recently, the anticancer potential of plant-derived compounds has gained considerable attention due to their low toxicity profiles and bioactive properties (13). One such promising plant is Nigella sativa, commonly known as black cumin or black caraway, a herbaceous annual plant from the Ranunculaceae family (14). It is widely cultivated across West Asia, East Africa, and the Middle East. The seeds of N. sativa have been traditionally used in medicine and are reported to possess antioxidant, antidiabetic, immunomodulatory, and anticancer effects (15). They are rich in proteins, fatty acids, carbohydrates, amino acids, alkaloids,

22

[43]

crude fiber, along with essential minerals like sodium, calcium, potassium, iron, and water-

soluble vitamins (16).The primary bioactive compound of black seed is Thymoquinone (TQ) [2-methyl-5-isopropyl-1,4-benzoquinone], first extracted by El-Dakhakhany (17). TQ has been widely studied for its anticancer, anti-inflammatory, and antioxidant properties. Techniques such as gas chromatography, high-performance liquid chromatography (HPLC), and differential pulse polarography are used for its quantification (18). Other active components of the seed include thymohydroquinone, dithymoquinone, and thymol (19). TQ's lipophilic quinone structure enables it to penetrate cellular and subcellular membranes, targeting transcription factors and kinases involved in the cancer microenvironment. It has demonstrated effectiveness in inhibiting proliferation, migration, invasion, and angiogenesis in cancer models.TQ's anticancer potential has been tested on several human lung cancer cell lines, including A549, NCI-H146, and NCI-H460, where it was found to significantly increase levels of pro-apoptotic markers such as p53, the Bax/Bcl-2 ratio, and caspases, while inhibiting angiogenesis by downregulating Gro-alpha and ENA-78, which are key pro-angiogenic factors (20,21,22).The A549 cell line, specifically, is a widely accepted in vitro model derived from human alveolar basal epithelial cells. It serves as an excellent system for NSCLC studies due to its well- characterized phenotype, responsiveness to apoptotic stimuli, and relevance in evaluating natural compound-based therapeutics.This background thus lays the foundation for exploring the apoptosis-inducing effects of Thymoquinone in A549 cells as a potential therapeutic approach for non-small cell lung cancer.

Chapter 2:- Review of Literature

Thymoquinone (TQ), a naturally occurring bioactive compound, has garnered significant interest for its therapeutic potential in treating various diseases, including lung cancer. Several studies have reported the pro-apoptotic effects of TQ in lung carcinoma models,

highlighting its promise as an anti-cancer agent (23).

In vivo studies by Gajendra et al. (2022) demonstrated that TQ effectively attenuated lung

carcinogenesis induced by Benzo(a)pyrene (B(a)P) in Swiss Albino mice. Administration of TQ at a dose of 20 mg resulted in improved body weight, reduction in lung weight, and a decreased mortality rate of 10% in B(a)P-induced lung cancer models (24). B(a)P-diol-epoxide (BPDE), an active metabolite of B(a)P, is a potent carcinogen capable of forming DNA adducts and causing chromosomal abnormalities, underscoring the severity of its carcinogenicity.

Further advancements in TQ delivery were reported by Khan et al. (2022), who fabricated pH-sensitive liposomes encapsulating TQ (TQPSL) for targeted therapy in B(a)P-exposed mice. The pH-responsive liposomes were designed to prevent premature drug release under physiological pH, enabling efficient TQ delivery in the acidic tumor microenvironment. Mice treated with TQPSL after 6-8 weeks of B(a)P exposure exhibited significant reductions in biochemical markers associated with lung cancer progression, including A, γ-GT, AHH, ADA, 5′-NT, and LDH. Importantly, pretreatment with TQPSL markedly inhibited lung cancer cell proliferation (25).

At the cellular level, TQ exerts its anti-cancer effects by modulating multiple signaling pathways. Yang et al. (2015) demonstrated that TQ suppresses proliferation, invasion, and migration of A549 lung cancer cells through inhibition of the ERK1/2 signaling pathway. Specifically, TQ reduced the phosphorylation of ERK1/2, leading to decreased expression of proliferative markers such as PCNA, cyclin D1, and matrix metalloproteinases MMP2 and MMP9, as well as downregulation of P16 and gelatinase activity (26). Additional studies using phytosomal formulations of TQ showed cell cycle arrest in the G2/M and pre-G1 phases and significant elevation in reactive oxygen species (ROS) levels, implicating oxidative stress as a mechanism of apoptosis induction (27).TQ also targets microtubule dynamics, a critical factor in maintaining cellular structure and mitosis. Direct interaction of TQ with tubulin polymers leads to their depolymerization, causing cell cycle arrest in the

G2/M phase (28). Furthermore, TQ has been shown to reduce levels of ENA-78 and GRO cytokines, which are involved in angiogenesis and neovascularization, thereby contributing to its anti-angiogenic effects. Annexin-V staining revealed a high percentage (73.5%) of

apoptosis in treated cells (29). Another notable mechanism involves TQ binding to G-quadruplex (G4) DNA structures at telomeric ends, resulting in cell cycle arrest at the G1 phase and increased cellular senescence (30).To enhance the bioavailability and efficacy of TQ, various nanoparticle-based delivery systems have been developed. PEGylated PLGA nanoparticles conjugated with transferrin

[3]

(TF-TQ-Np) demonstrated improved anti-cancer activity by upregulating tumor-suppressive microRNAs, including miR-34a and miR-16, which inhibit the anti-apoptotic protein Bcl-2, thereby promoting apoptosis in A549 cells (31). In a similar vein, Upadhyay et al. (2021) reported that transferrin-conjugated nanoparticles co-delivering TQ and gefitinib effectively overcame gefitinib resistance in A549 cells (32). Additionally, phytophospholipid nanovesicles combined with scorpion venom enhanced TQ-induced apoptosis, increasing the expression of key pro-apoptotic genes such as p53, caspase-3, and Bax while modulating inflammatory markers like NF-κB and TNF-α, suggesting promising therapeutic potential in lung adenocarcinoma treatment (33).

Combination therapies involving TQ and other chemotherapeutic agents have demonstrated synergistic effects in inducing apoptosis. For instance, the combination of TQ with quercetin (TQ+Qu) in A549 cells resulted in 50.9% early apoptosis, surpassing the apoptosis rates induced by TQ (40.6%) or quercetin alone (33.3%). This combination also increased both early and late apoptosis phases and downregulated pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression, highlighting enhanced cytotoxicity (34). Similarly, TQ combined with cisplatin (CDDP) significantly reduced tumor volume and weight in mouse xenograft models in a dose-dependent manner. This combination also modulated NF-κB signaling pathways and inhibited Polo-like kinases, key regulators of mitotic progression (35).

Moreover, Dera et al. (2020) observed that 29 the combination of indirubin-3-monoxime (TQ decreased cell viability, increased apoptosis, and caused G0 phase cell

cycle arrest in vitro. This combination also impaired metastatic potential and tumor growth in vivo, downregulating oncogenic markers such as p-AKT, p-mTOR, caspase-3, p53, and NF-κB, further supporting the synergistic anti-carcinogenic effects (36). Similarly, the TQ and resveratrol combination reduced cellular viability in A549 cells, accompanied by increased expression of apoptotic genes including Bax, Fas, FasL, p53, and caspases 3, 8, and 9. This combination also elevated levels of p53, p27, and p21 and disrupted mitochondrial membrane integrity, potentiating apoptotic pathways (37)

Chapter: 3 MATERIALS AND METHODS:-

3.1 Cell Culture:

The primary goal of this procedure was to establish, maintain, and propagate a healthy population of A549 cells (a non-small cell lung carcinoma line) under optimal in vitro conditions to serve as the biological model for evaluating the apoptotic response to thymoquinone.

Materials and Reagents

- A549 cell line
- Dulbecco's Modified Eagle Medium (DMEM), high glucose formulation

- Fetal Bovine Serum (FBS), heat-inactivated
- Penicillin-Streptomycin solution (100×)

□ 0.25% Trypsin-EDTA

□ Phosphate Buffered Saline (PBS), pH-adjusted to 7.4

□ Sterile T-flasks (T-25) and T-75)

□ Dimethyl sulfoxide (DMSO)

□ CO_2 Incubator (37°C, 5% CO_2 atmosphere)

□ Class II Biosafety Cabinet

□ Inverted Phase Contrast Microscope

Reagent Preparations

Complete Culture Medium

□ Combine DMEM with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin.

□ Mix gently and store at 4°C. Pre-warm to 37°C before use.

Phosphate Buffered Saline (PBS) – 1 L

□ Sodium chloride: 8.0 g

□ Potassium chloride: 0.2 g

□ Disodium hydrogen phosphate: 1.44 g

□ Potassium dihydrogen phosphate: 0.24 g

□ Adjust final pH to 7.4, sterilize by autoclaving.

[5]

Protocol

Thawing of A549 Cells

□ Rapidly thaw cryopreserved vials in a 37°C water bath (1–2 minutes).

□ Decontaminate the outer surface with 70% ethanol before placing in the biosafety hood.

□ Gently transfer the thawed contents into 5 mL of warm complete medium.

□ Centrifuge at 1000 rpm for 5 minutes to remove DMSO.

□ Discard the supernatant and resuspend cells in fresh medium.

□ Transfer the suspension into a sterile T-25 flask and incubate under standard culture conditions.

Routine Culture Maintenance

- Inspect cells daily for morphology and confluency.
- Replace old medium every 48–72 hours or sooner if media color change suggests nutrient depletion.
- To replace the medium:
  - Aspirate spent media without disturbing the monolayer.
  - Add pre-warmed complete medium gently to avoid detaching adherent cells. Sub-Culturing of Cells
- Conduct passaging once the monolayer reaches approximately 70–80% confluency.
- Remove medium and rinse gently with sterile PBS.
- Add 1 mL of Trypsin-EDTA to detach cells and incubate at 37°C for 2–3 minutes.
- Observe under microscope until detachment is complete.
- Add 4 mL fresh complete medium to neutralize the enzyme.
- Transfer to a tube, spin down at 1000 rpm for 5 min.
- Discard the supernatant and re-suspend the pellet in 5 mL of growth media.
- Re-seed into new flasks at a ratio of 1:4 or 1:5 as needed.

[6]

3.2 MTT Assay

The aim of the MTT assay was to evaluate the cytotoxic and anti-proliferative effects of Thymoquinone on A549 human lung carcinoma cells by assessing cell metabolic activity as an indirect indicator of cell viability following treatment.

30 The MTT assay is a colorimetric technique [53] used to assess the viability and proliferation of cells based on their metabolic activity. The assay employs 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow-colored, water-soluble tetrazolium salt.

Viable cells possess active mitochondrial dehydrogenase enzymes that reduce MTT into insoluble purple formazan crystals. This reduction occurs only in metabolically active (living) cells, while dead or non-proliferating cells cannot perform this transformation. The formazan is then dissolved using an organic sol 23 is directly proportional to the number of yield a colored solution, the intensity of which 24 measured using a spectrophotometer or viable cells. The absorbance is quantitatively micropla at a wavelength of 570 nm, with a reference filter often set at 630–690 nm to subtract background.

This assay provides a reliable estimation of cell survival, cytotoxic effects of drugs, and potential anti-cancer activity of compounds such as thymoquinone.

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Dulbecco's Modified Eagle Medium (DMEM), with 10% FBS and 1% Pen-Strep
- Thymoquinone (TQ)
- MTT reagent (Sigma-Aldrich or equivalent)
- Dimethyl sulfoxide (DMSO)
- 96-well flat-bottom tissue culture plates
- CO_2 incubator
- Inverted phase contrast microscope
- Microplate reader or ELISA plate reader (570 nm)

[7]

[55]

Reagent Preparation

MTT Stock Solution (5 mg/mL)

□ Dissolve 5 mg of MTT in 1 mL of sterile PBS (filter-sterilized using a 0.22 µm filter).

☐ Store in the dark at 4°C for up to 2 weeks.

DMSO (Formazan Solubilization Solution)

☐ Use analytical grade DMSO directly.

☐ Store at room temperature, protect from moisture.

Protocol

Pre-treatment Setup

☐ Seed A549 cells into a 96-well plate at a density of 5×10^3 to 1×10^4 cells per well in 100 µL complete DMEM.

☐ Incubate the plate overnight at 37°C in a humidified incubator with 5% CO_2 to allow cell attachment.

Treatment with Thymoquinone

☐ Prepare different concentrations of thymoquinone (e.g.0.5,1,1.5,2,2.5,3,3.5,4,4.5,5,7,8,9 10 ug/ml) in complete medium.

☐ Replace the old medium in each well with 100 µL of TQ-containing medium.

☐ Include untreated control wells (only medium) and vehicle control (e.g., DMSO without TQ).

☐ Incubate for 24, 48, and/or 72 hours depending on experimental design. MTT Addition

☐ After the treatment period, add 10 µL of MTT stock solution (5 mg/mL) into each well to achieve a final concentration of 0.5 mg/mL.

☐ Incubate the plate for 3–4 hours at 37°C. During this time, purple formazan crystals will form in viable cells.

Solubilization and Reading

☐ Carefully remove the medium from each well without disturbing the crystals.

☐ Add 100 µL of DMSO to each well to dissolve the formazan crystals.

☐ Shake the plate gently for 10–15 25 minutes at room temperature in the dark to ensure complete solubilization.

[57]
[
8
]

- Measure the absorbance at 570 nm using a microplate reader.
- A reference wavelength of 630 nm may be used to subtract background absorbance.

3.3 Cell Cycle Analysis by Flow Cytometry

The objective of this experiment was to determine the effect of Thymoquinone (TQ) treatment on the distribution of A549 lung carcinoma cells across different phases of the cell cycle (G_0/G_1, S, and G_2/M phases) using propidium iodide (PI) staining followed by flow cytometric analysis.

Cell cycle analysis using flow cytometry allows for the quantification of DNA content in individual cells, which reflects their progression through the cell cycle. In this assay, cells are stained 9 with propidium iodide (PI), a fluorescent intercalating agent that binds stoichiometrically to DNA. Since DNA content doubles as a cell progresses from G_1 to G_2/M phase, PI fluorescence intensity directly correlates with the amount of DNA present. The protocol involves:

- Fixing cells in ethanol to permeabilize the membrane.
- Treating with RNase A to degrade RNA (which also binds PI and would otherwise confound results).
- Staining DNA with PI.

Using flow cytometry, cells in G_0/G_1 phase (2N DNA), S phase (synthesizing DNA), and G_2/M phase (4N DNA) are distinguished based on their fluorescence intensity. 9 The percentage of cells in provides insight into cell cycle arrest or progression induced by TQ treatment.

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Complete DMEM with 10% FBS and 1% Pen-Strep

- Thymoquinone (TQ)

☐ 1X Phosphate Buffered Saline (PBS)

☐ Ice-cold 70% ethanol

☐ RNase A (10 mg/mL)

[9]

☐ Propidium iodide (PI, 1 mg/mL stock)

☐ FACS tubes

☐ Flow cytometer (with appropriate red fluorescence detector, e.g., FL2 or FL3 channel)

☐ Microcentrifuge and cooling centrifuge

Reagent Recipes

☐ 70% Ethanol: Mix 70 mL absolute ethanol with 30 mL sterile distilled water; chill at −20°C.

☐ PI Staining Solution (for 1 mL):

o PI: final concentration of 50 µg/mL

o RNase A: final concentration of 100 µg/mL

o Dilute both in 1X PBS, prepare fresh or store aliquots at −20°C.

Protocol

Cell Treatment and Harvesting

☐ Seed A549 cells in 6-well plates and allow them to adhere overnight.

☐ Treat cells with desired concentrations of Thymoquinone for 24 or 48 hours.

☐ After treatment, collect both floating and adherent cells by trypsinization and pooling.

Fixation

☐ Wash the cell pellet once with ice-cold PBS.

☐ Resuspend cells in 0.5 mL PBS.

☐ Add 4.5 mL of pre-chilled 70% ethanol dropwise while vortexing gently.

☐ Incubate at −20°C for at least 2 hours or overnight for proper fixation. RNA

Removal and DNA Staining

☐ Centrifuge fixed cells at 300 × g for 5 minutes and discard the ethanol.

☐ Wash the pellet twice with PBS to remove residual ethanol.

[10]

☐ Resuspend cells in 500 µL PI staining solution (containing PI and RNase A).

☐ Incubate in the dark at 37°C for 30 minutes.

Flow Cytometry Analysis

☐ Transfer stained cells into FACS tubes.

☐ Filter through a nylon mesh or cell strainer to remove clumps (optional but recommended).

☐ Analyze samples using flow cytometry.

☐ Acquire at least 10,000 events per sample.

☐ Evaluate data using software like FlowJo or BD CellQuest to determine the percentage G_0/G_1, S, and G_2/M phases.

3.4 Annexin V/Propidium Iodide (PI) Assay

The aim of this experiment was to evaluate the extent of apoptosis induced by Thymoquinone (TQ) in A549 human lung cancer cells by using Annexin V-FITC/Propidium Iodide (PI) dual staining followed by flow cytometric analysis. This assay helps distinguish between live, early apoptotic, late apoptotic, and necrotic cells.Apoptosis, or programmed cell death, involves early changes in the plasma membrane, one of which is the externalization of phosphatidylserine (PS) from the inner to the outer leaflet. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, thus detecting cells undergoing early apoptosis.Propidium Iodide (PI) is a membrane-impermeable DNA-binding dye that only penetrates cells with compromised membrane

[61]

integrity—marking late apoptotic or necrotic cells. When used together:

Cell Status

Annexin V-FITC PI

Live cells

–

–

Early apoptosis

+

–

Late apoptosis

+

+

Necrosis

–

+

Table 1 :- Cell Status against staining with Annexin V-FITC and PI

By measuring fluorescence from both Annexin V-FITC (green) and PI (red), flow cytometry can classify and quantify cells in each category.

[11]

Materials and Reagents

Reagents and Chemicals

- A549 human lung carcinoma cells
- Thymoquinone (TQ)
- Complete DMEM (with 10% FBS and 1% Pen-Strep)
- 1X Phosphate Buffered Saline (PBS) [63]
- Annexin V-FITC Apoptosis Detection Kit (includes Binding Buffer, Annexin V-FITC, and

PI)

☐ FACS tubes

☐ Trypsin-EDTA

☐ Flow cytometer (with FL1 and FL3/FL2 channels for FITC and PI respectively) Reagent Recipes

☐ 1X Binding Buffer (if not provided in kit):

o 10 mM HEPES, pH 7.4

o 140 mM NaCl

o 2.5 mM $CaCl_2$

Filter-sterilize and store at 4°C. Always prepare fresh before use if homemade.

Protocol

Cell Treatment and Harvesting

☐ Seed A549 cells in 6-well plates and incubate overnight for adherence.

☐ Treat cells with different concentrations of Thymoquinone for 24 or 48 hours.

☐ Collect both floating and adherent cells by trypsinization.

☐ Wash cells twice with cold 1X PBS by centrifuging at 300 × g for 5 minutes. Staining for Apoptosis Detection

☐ Resuspend ~1 × 10^5 to 1 × 10^6 cells in 100 µL of 1X binding buffer.

☐ Add 5 µL Annexin V-FITC and 5 µL PI to the cell suspension.

☐ Gently vortex and incubate 15 in the dark at room temperature for 15 minutes.

☐ After incubation, add 400 µL 16 1X binding buffer to each tube.

☐ Keep the tubes on ice and analyze samples Flow within 1 hour by flow Cytometric Analysis

☐ Set flow cytometer for FL1 (FITC) and FL2/FL3 (PI) detection.

☐ Collect data for at least 10,000 events per sample.

☐ Gate the cell population properly and use quadrant analysis 9 to determine the

percentage

- Live (Annexin−/PI−)
- Early apoptotic (Annexin+/PI−)
- Late apoptotic (Annexin+/PI+)
- Necrotic (Annexin−/PI+)

3.5 WESTERN BLOTTING

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells.

The technique uses three elements to accomplish this task:

(1) separation by size

(2) transfer to a solid support

(3) marking target protein using a proper primary and secondary antibody to visualize.

A synthetic or animal-derived antibody (known as the primary antibody) is created that recognizes and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein.Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band

should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

PROCEDURE :

Protein Isolation :

RIPA Buffer recipe

Table 2 :- RIPA Buffer composition

For protein isolation or protein purification there are the following steps –

1) ➢ Firstly, appropriate cells are cultured in DMEM media buffer and store in an Eppendorf.

2) ➢ Then it is centrifuged at 3000 rpm for 3 minutes in 4 degree C. The pellet should be collected and resuspend in a Eppendorf with DMEM medium buffer.

3) ➢ Centrifuge at 3000 rpm for 3 minutes in 4 degree C.

4) ➢ Pipette out the supernatant and add PBS wash buffer to the collected pellet.

5) ➢ Then centrifugation has to be done at 3000 rpm for 3 minutes in 4 degree C .

6) ➢ Pipette out the wash buffer (supernatant) PBS .

7) ➢ The pellet is collected in an Eppendorf and mixed with RIPA buffer for lysis of the cell and extraction of protein

8) ➢ Store in ice box for 15 minutes at 4 degree C.

9) ➢ The RIPA buffer containing the pellet is again resuspended and again store in ice box for 15 minutes at 4 degree C.

10) ➢ Repeat the process of resuspension and storing for three times.

11) ➢ Finally, centrifugation has to be done at 13000 rpm for 25 minutes at 4 degree C.

12) ➢ Here, the pellet is the cell debris which has to be removed . The supernatant is the extracted protein. Obtain the protein for the next step – Protein Estimation.

2) Protein Estimation :

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents Required :

a) BSA Protein Assay Reagent A : 2% sodium carbonate in 0.1 N sodium hydroxide.

b) BSA Protein Assay Reagent B : 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate. Prepare fresh by mixing stock solutions.

c) BSA Kit : BSA 1 mg/ml , BSA 3 mg/ml , BSA 5 mg/ml.

d) Distilled water

e) Protein 1 and Protein 2.

Procedure:

➢ Mix (100×n) µl [n = no. of wells] of Reagent A and (2×n) µl of Reagent B. So for 6 wells there should be (6×100) µl i.e. 600 µl of reagent A and (2×6) µl i.e. 12 µl.

➢ Take a well plate and mark 6 wells in a row as Blank, sample 1, sample 2, sample 3, Unknown 1, Unknown 2.

Blank

Sample 1

Sample 2

Sample 3

Unknown 1

Unknown 2

Reagent

A+B

100 µl

100 µl

100 µl

100 µl

100 µl

100 µl

Distilled

water

15 µl

12.5 µl

12.5 µl

12.5 µl

12.5 µl

12.5 µl

BSA /

Protein

2.5 µl

(1mg/ml) BSA

2.5 µl

(3mg/ml) BSA

2.5 µl

(5mg/ml) BSA

2.5 µl

Protein 1

2.5 µl

Protein 2

Table 3:- BCA protein estimation assay

□ Incubate the well plate in a dark environment for at least 30 minutes.

□ Check the absorbance in Multiscan Go.

[15]

Separation of protein by SDS-PAGE:-

Table 4:- Stacking gel recipe

Table 5:- Separating gel recipe

[72]

Table 6:- Sample loading buffer

Table 7:- Electrophoresis Running Buffer

[16]

Table 8:- Sample preparation Buffers

Table 9:- Gel casting Reagents

[17]

Gel Casting :

❖ Putting together the parts you'll need to cast the gel: a tiny glass plate, a green casting frame, a casting stand, and a thick glass plate with 1 mm spacers connected.

[73]

❖ When the green casting frame is set on the bench with the green "feet" securely pressed to the bench, the clamps should be open (perpendicular to the frame) and facing the worker.

6 the two gel plates in the frame

❖ Putting (a fake plate is appropriate if only one gel is

needed). The BioRad logo should be when you insert the thicker spacer plate into the casting frame with the spacers facing the person and the "UP" arrows up. The short glass plate is inserted into the casting frame's front. The plates ought to be separated by a gap.

❖ Pushing the frame's two gates out to the sides will secure the plates in

❖ The casting frame's gates should be facing you as you clamp it onto the casting stand using glass plates.

❖ By pipetting a tiny 6 amount of deionized water into the space between the completed plates in the casting stand, you may verify that they are securely sealed. It is ready to create the gels if the glass plates retain water and do not leak. Holding the entire casting platform over a sink or liquid waste container allows you to pour out the water. Any remaining water can be absorbed using tissues or paper towels. Disassemble dry the then reassemble if the gel leaks.

❖ Fill the gap between the two plates with the resolving gel solution using a plastic transfer pipette until it is slightly higher than the green clamps on the gel

❖ Add butanol via a transfer pipet so that it runs smoothly over the polyacrylamide mixture's surface. When the polyacrylamide gel polymerizes, the water layer guarantees that its surface will be level.

❖ After polymerization is finished, tilt the resolving gel sideways and use a tissue to scrape the butanol off the top of the gel.

❖ Between the two glass plates, pipette the stacking gel over the resolving gel using a transfer pipette. Pour in enough stacking solution to just cover the little plate.

❖ Quickly but carefully lower the comb into place, taking care to avoid creating air bubbles. (The comb should have the Bio Rad logo facing you.) It's okay if some solution is forced out of the gel when the comb is added. Should air bubbles form beneath the comb, take it out and relocate it. [18]

❖ Carefully remove the comb from the spacer gel.

❖ With the short plate pointing inward, position the gel cassette 4 sandwich against the gasket on one side of the electrode assembly after removing the casting frame. 20 On the other side of the electrode assembly, place a buffer dam or a second gel cassette up against the gasket.

❖ Secure the green clamps to the electrode assembly's sides.

❖ The chamber should be lowered into the electrophoresis tank.

❖ Use the Tris-glycine running buffer to fill the gap between the two gels. This creates the electrophoresis top chamber.

❖ Fill the outer (lower) chamber with Tris-glycine running buffer (also known as electrophoresis buffer) 4 until the level is high enough to cover the platinum wire in the electrode

❖ Make a protein and loading dye combination. To run in a single well, prepare 20 µl of protein and 5 µl of loading dye. We must appropriately combine 5×4 µl of loading dye with 20×4 = 80 µl of protein in order to make loading protein for 4 wells.

❖ Place a micropipette containing 25 µl of the prepared protein-loading dye mixture into each well.

❖ Samples should be loaded gradually and given time to equally settle on the well's bottom.

Running Gel :

❖ 4 Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).

❖ Turn on the power supply. Run the gel at 90V until the sample protein reaches the bottom of the stacking gel and then run the gel at a constant voltage of 120 V resolving gel. Run the gel until the blue dye front nearly reaches the bottom

Blotting :

In order to make protein 3 accessible to antibody detection, they are moved from within

the gel onto a membrane made of nitrocellulose or poly viniledene di fluoride (PVDF). [2] Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol. The transfer procedure has been performed for the quantitative recovery of proteins from SDS-containing polyacrylamide gels.

[19]

Procedure:-

❖ Cut the gel with a gel cutter from the gel plate and cut into a size for transferring the bands to the membrane.

❖ Take the PDVF membrane the and wet the PDVF membrane in for activation of membrane.

❖ Wet the sponge and filter papers

❖ Make a sandwich .

❖ Carefully place the cathode plate onto the stack. Press to engage the latches with the guide posts without disturbing the gel/nitrocellulose stack.

❖ Added [2] transfer buffer to the apparatus, and ensure that the sandwich was covered with the buffer.

❖ [13] All components in contact with the slab gel were prewetted in the electrode (Transfer Buffer) composed of 20 mM Tris base, 150 mM glycine, and 20% methanol. solution

❖ This solution should be degassed briefly under vacuum before use. Here, the method for transferring proteins is called electroblotting, where electric current was used to pull proteins from the gel into membranes within the machine Trans-Blot Turbo Transfer System by BIO RAD.

Binding of antibody to nitrocellulose-immobilized proteins:

❖ [2] Blocking is a very important step of western blotting, as it prevents antibodies from binding to the membrane nonspecifically. [77]

❖ Immediately following transfer, the nitrocellulose sheet is immersed in a mild blocking

solution containing non fat dried milk powder in Tris Buffer Saline (TBS) along with a detergent, Tween 20 (together called TBST) and incubated at 40°C for 1hr on a rocking platform to prevent the non-specific 3 interactions between the membrane and the antibody used for the detection of the target protein.

❖ The nitrocellulose was then washed with rocking for 10 min in Tris-saline for three times. The sheet is immersed in Primary antibody solution on a rocking platform overnight at 4°C.

❖ Then next day the sheet was kept in room temperature for 1 hour on rocking condition. then sheet was again washed with rocking for 10 min in Tris-saline for three times.

[20]

Table 10:- Transfer Buffer Recipe

Table 11:- TBS recipe

Fig 1:- Wet transfer setup for Western blotting

[21]

❖ After that Secondary antibody was added to the sheet and placed on a rocking platform at room temperature for 2 hr.

[78]

❖ 2 Washing is very important as it minimized background and removes unbound antibody. However, the membrane should not be left to wash for a really long time, as it can also reduce the signal.

❖ The nitrocellulose sheet was then washed with rocking for 10 min in Tris-saline for three times.

❖ After that, the developing solution (containing Luminol and p-Coumaric acid) was applied on the nitrocellulose sheet, and incubated in dark room on rocking condition for 2 to 3 mins, which will form dipharmazone, a blue coloured complex; catalysed by the enzyme alkaline phosphatase (ALP), which was present in the Secondary antibody, i.e.; wherever Secondary antibody is present, blue coloured band will develop there.

❖ The sheet was then scanned in Chemi-Doc XR+ by BIO RAD and the images are taken and saved.

4.1 A549 cell line

Fig 2:- A549 Cell line

[1] A549 cells are adenocarcinomic human alveolar basal epithelial cells, and constitute a cell line that was first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male. The cells are used as models for the study of lung cancer and the development of drug therapies against it.

Characteristics: A549 cells, as found in the lung tissue of their origin, are squamous and responsible for the diffusion of some substances, such as water and electrolytes, across alveoli. If A549 cells are cultured in vitro, they grow as a monolayer; adherent or attaching to the culture flask. The cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids, which are important to maintain membrane phospholipids. A549 cells are widely used as a type II pulmonary epithelial cell model for drug metabolism and as a transfection host. When grown for a sufficiently long time in cell culture, A549 cells may begin to differentiate, as signaled by the presence of multilamellar bodies.

[23]

4.2 A549 cell viability analysis on the application of TQ through MTT Assay:-

(a)

(b)

[81]

Figure 3 - Analysis of percentage of cell survival by MTT assay on A549 cells on exposure to

TQ (a) in increasing TQ concentrations (0-10.5/ml) of TQ after 24 h of incubation (b) at different time points (1,2, 4, 6, 8,12, 24, 36, 48 and 72 h), in an increasing manner.

As shown in Fig., exposure of A549 cells (a) in increasing TQ concentrations (0-10µg/ml) of TQ for 24 h, decreased the percentage of cellular viability in a concentration/dose-dependent manner. The IC50 concentrations (producing half maximal inhibition) at 24 h were between 2.5 and 5µg/ml of TQ for all cells, and the IC50 was 5µg/ml of TQ. In Fig. (b), at different (increasing) time points (1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h), reduced the percentage of cell survival in a time-dependent manner. It is also evident that the half maximal survival (50%) of cells occurs at 24 h.

[24]

4.3 Cell cycle analysis:-

(a) 1: Untreated (a) 2: TQ (5µg/ml). (b)

Fig 4: Analysis of percentage of cell death or cellular toxicity by flow-cytometric cell cycle analysis on A549 cells, (a) 1: one control set up (without any treatment), (a) 2: along with dose of TQ (5µg/ml). (b) Graphical representation of Cell cycle analysis.

[83]

Cell cycle analysis was conducted to determine the effect of thymoquinone (TQ) at 5 µg/mL on A549 lung cancer cells. The data showed a significant increase in the sub-G1

population, indicating DNA fragmentation and apoptosis. This suggests that TQ induces apoptotic cell death rather than arresting cells at a specific checkpoint. A slight decrease in the S-phase population was also observed, implying reduced DNA synthesis and inhibition of cell proliferation. Minor changes in the G2/M phase suggest that TQ does not strongly interfere with mitotic progression. The prominent accumulation of cells in the sub-G1 phase aligns with previous findings that TQ triggers apoptotic pathways. These results support
the notion that TQ at 5 µg/mL disrupts cell cycle progression and promotes apoptosis in A549 cells. Thus, TQ demonstrates potent anti-proliferative and pro-apoptotic effects, reinforcing its potential as an effective anticancer agent for lung cancer therapy.

[25]

4.4 Determining cellular apoptosis using Annexin V/ PI Assay:

(a) (b)

Fig 5:- The representation of apoptotic and necrotic populations of cells treated with indicated doses of TQ in A549 cells using Annexin V/PI in flow cytometry. (a) One control set up (without any treatment), (b) along with a dose of TQ (5µg/ml).
A549 cells were labelled with PI and Annexin V-FITC and analysed on a flow cytometer Dual parameter – quadratic dot plot of Annexin V (y-axis) versus PI fluorescence (x-axis) has been shown in logarithmic fluorescence intensity. Annexin v is a marker of apoptosis, while PI is a marker for necrosis.

[85]

The treatment of A549 cells with TQ increased the rate of apoptotic cell death. This finding

thereby established the fact that the TQ showed apoptotic cell death in lung cancer cells at dose (5µg/ml) as compared to the control set up (with no treatment). Quantitative evaluation of apoptosis by annexin V/PI test indicated that the ratios of apoptotic cells

significantly increased after TQ treatment. Using flow cytometry, the annexin V–FITC test confirmed the apoptotic potential of TQ against A549 cancerous cells.Cells 8 that were propidium iodide (PI) negative and Annexin V negative (4th quadrant) are considered healthy, cells, PI negative and Annexin V positive cells (1st quadrant) are considered apoptotic, and cells that are positive to both PI and Annexin V (2nd quadrant) considered necrotic .

[26]

4.5 Analysing the expression pattern of protein through Western Blot:

PROTEINS A549 kDa

(a) (b)

Fig. 5: Western blot analysis showing expression levels of p53, Bax and Bcl-2 proteins in A549 cells, both in – (a) control set up and after TQ treatment with β- actin as the loading control.(b) Graphical representation of the same.

p53 and Bax are important pro-apoptotic regulatory proteins, while Bcl-2 functions as an anti-apoptotic protein that inhibits cell death. Assessing their expression is vital to understanding the apoptotic potential of therapeutic agents. Western blotting is a standard technique used to measure protein expression. In this study, we aimed to explore the molecular mechanism behind the anti-migratory effect of thymoquinone (TQ) on A549 lung

[87]

cancer cells, with a focus on p53, a known tumor suppressor gene. TQ has been reported to upregulate p53, which can further activate downstream targets such as miR-34a, known

to promote apoptosis. We performed Western blot analysis to examine the expression levels of p53, Bax, and Bcl-2 in TQ-treated and untreated A549 cells. The results showed a

clear increase in p53 and Bax expression, along with a notable decrease in Bcl-2 levels upon TQ treatment, indicating activation of the intrinsic apoptotic pathway. These findings were further supported by immunofluorescence imaging. β-actin was used as a housekeeping protein and loading control to validate the consistency of protein loading. Altogether, the results suggest that TQ promotes apoptosis in A549 cells by upregulating p53 and Bax, while downregulating Bcl-2, supporting its potential as an anti-cancer agent.

[27]

Chapter 5 : -Conclusion

Determining the cellular viability with MTT assay as shown in Fig.1 revealed decreased cellular viability with increasing TQ concentrations (0-10ug/ml) for 24hrs. (a)The half maximal inhibitory concentration , IC50 value of the treated cells was between 2.5 ug/ml and 5ug/ml. (b) A reduction in the percentage of treated cell was observed at various time points(1, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 72 hrs). The IC50 value was dominant at 24hrs.To find out cellular apoptosis on inducing our NSLC cell line, A549 with TQ we performed flow cytometric analysis. Our treated and untreated A549 cell line was tagged using FITC-Annexin V propidium iodide (PI) and FITC-Annexin V and was then analyzed on a flow cytometer. The depiction of logarithmic fluorescence intensity was shown by the dual parameter – quadratic dot plot of FITC fluorescence (x-axis) versus PI fluorescence (y-axis). Annexin v is a marker of apoptosis, while PI is a marker for necrosis. Treating the A549 cells with thymoquinone resulted in increase in the apoptosis rate. Thereby, this finding determinates the fact that the TQ showed apoptosis in our cell line A549 at a dosage (5µg/ml, Fig.2) as compared to the control set up (with no treatment). Annexin V/PI test was used to quantitatively assess apoptosis. The results showed that following TQ treatment, the ratios of late-stage and early-stage apoptotic cells considerably rose from 8.01% and 1.13% (in the control setup, without treatment) to 11.2% and 42.89%, respectively. Hence, the apoptotic potential of TQ against the lung cancer cell line, A549

was proven using flow cytometry through the Annexin V-FITC test.Flow cytometric cell cycle phase distribution of the TQ treated A549 was performed to check the cytotoxic potential of TQ. We observed significant A549 cell death on treating the cells with TQ (5 g/ml). Prominent cellular cytotoxicity was observed at very low dosage 5 g/ml. Additional cell cycle analysis was performed, and TQ was found in inhibiting the molecules involved in S-phase following sub-G1 phase arrest occurred. To monitor the expression levels of the apoptotic gene p53, we presumed its participation in the investigation of TQ's antimigratory effects on A549 cells. It is a well-known fact that TQ induces cells to up-regulate their p53 levels. We performed Western blotting to check the expression pattern of p53, Bax and Bcl-2, keeping β-actin as the loading control or as housekeeping gene control.

[28]

protein on treating the A549 cells with TQ by keeping a control setup without treatment. This clearly indicated the induction apoptosis by p53 dependant pathways in the cells.The treatment of lung cancer cells with the bioactive compound derived from N.sativa, Thymoquinone (TQ) has shown to reduce cellular viability along with induced cell death in the A549cells. Our study has strongly reported the usage of TQ in exhibiting strong specificity towards the migration of cancer cell, with it being less toxic to our normal body cells. Our findings have also demonstrated the role of TQ in cell cycle arrest at the Sand sub G1 phase. Our current report has also focussed on delineating the pathways involved in the apoptosis of cancer cells. We found out that the underlying pathways involved in the apoptosis of A549 lung cancer cells, treated with TQ recruits the p53 dependent apoptotic pathways. The downregulation of the antiapoptotic gene Bcl2 and the upregulation of Bax were reported in our study. As a whole, [90] conclude that this lipophillic bioactive compound, TQ can be regarded as chemotherapeutic and chemopreventive substance in

inducing cellular apoptotsis and viability of the NSCLC cell line, A549 cells.

[29]

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